AD_____

Award Number: DAMD17-01-2-0071

TITLE: Development of Potent Orally Active Agents for Prevention and Treatment of Poxvirus Infection

PRINCIPAL INVESTIGATOR: Karl Y. Hostetler, M.D.

CONTRACTING ORGANIZATION: Veterans Medical Research Foundation
San Diego, CA 92161

REPORT DATE: November 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2003	Annual (1 Oct 2002 - 1 Oct 2003)

4. TITLE AND SUBTITLE

Development of Potent Orally Active Agents for Prevention and Treatment of Poxvirus Infection

5. FUNDING NUMBERS

DAMD17-01-2-0071

6. AUTHOR(S)

Karl Y. Hostetler, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Veterans Medical Research Foundation
San Diego, CA 92161

E-Mail: khostetler@ucsd.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Oral antiviral drugs would be highly desirable for the prevention and treatment of smallpox biowarfare or bioterrorism casualties. During the second year of this grant, we concentrated on obtaining in vivo data on the efficacy of hexadecyloxypropyl-cidofovir (HDP-CDV) and octadecyloxypropyl-CDV (ODE-CDV) in animal models of orthopoxvirus disease including. In collaboration with Dr. John Huggins of USAMRIID, Dr. Mark Buller of St. Louis University and Dr. Earl Kern of the University of Alabama, we found that HDP-CDV and ODE-CDV were highly protective in lethal models of cowpox, vaccinia and ectromelia as well as murine CMV. We synthesized 40 gram lots of HDP-CDV and ODE-CDV and supplied them to the company who has been selected by NIAID to do the preclinical development of these two compounds for the prevention and treatment of smallpox. We discovered a new series of highly active CDV analogs lacking the linker moiety. We also synthesized 1-O-hexadecyloxypropyl-(S)HPMPA, a novel ether lipid prodrug of HPMPA and and HDP-PMEG and several related analogs. In cells infected with cowpox or vaccinia viruses, HDP-(S)HPMPA and HDP-PMEG inhibited the replication of all test viruses with a 50% effective concentration (EC_{50}) between 0.019 and 0.12 μM while the unmodified PMEG and HPMPA showed EC_{50} values between 2.7 and 10.6 μM During the 03 year we will focus on obtaining animal data in orthopoxvirus infected animals with HDP-(S)HPMPA and HDP-PMEG.

14. SUBJECT TERMS

Virus, cidofovir, prodrugs, animal models

15. NUMBER OF PAGES

116

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

INTRODUCTION

Smallpox (variola major) is estimated to have killed a billion persons over the past 200 years. Vaccination eliminated the disease from the earth in the late 1970s (WHO) but stocks of smallpox have been retained by the US and Russia at safe locations. However, rogue states or terrorist groups may also have stocks of smallpox which could be used in biowarfare or bioterrorism. In the late 80s, Russia produced tons of variola, India-1, for use in long range ICBMs (Alibek) and it is uncertain what has become of those stocks and the thousands of persons trained to produce the variola virus.

The US population has not been vaccinated since the late 70s and is therefore highly susceptible to infection. Although sufficient vaccine has been identified for use in a national emergency, vaccine reactions may be severe in persons with weak immune systems (AIDS, Cancer or Organ/bone marrow transplants) or in patients with skin conditions like eczema and atopic dermatitis. For these reasons, it would be useful to have a safe and effective oral agent which could be used to prevent or treat variola infection for use in persons in whom vaccination would be to dangerous.

Furthermore, interleukin-4 (IL4+) positive strains of poxvirus have been reported by Australian researchers which may be able to circumvent vaccine immunity. This genetically engineered approach, if applied to variola, could produce a smallpox virus which would be effective in causing illness and death, even in vaccinated persons. Therefore, an effective antiviral countermeasure would be highly useful, particularly one which works orally and could be self administered easily. Our compounds, HDP- CDV and ODE-CDV have been shown to be fully active against these IL-4 modified strains of ectromelia (Buller and Hostetler, in press, 2003).

In the first year of this project, we synthesized over 20 highly active and selective orally active derivatives of cidofovir and cyclic cidofovir and assessed their in vitro activity and cytotoxicity. Some of the more promising agents have been successfully tested in vivo in lethal models of poxvirus disease (HDP-CDV; ODE-CDV, OLP-CDV and OLE-CDV). We have also identified two other classes of highly active poxvirus drugs which are NOT based on cidofovir. These new compounds are 8.8 to 73 times more active than HDP-CDV *in vitro* against vaccinia virus and 5 to 32 times more active than HDP-CDV versus cowpox virus.

In the 02 year, we concentrated on large scale synthesis and provided 4 compounds: HDP-, ODE-, OLP and OLE-CDV to our collaborators for study in lethal challenge models of poxvirus in mice. We also synthesized 40 grams of HDP-CDV and ODE-CDV and provided this to Chimerix Inc., the company selected to do the development for smallpox.

BODY:

Specific Goal #1: To Carry out Structure-Activity Assessments of the *in vitro* Activity and Selectivity of Ether Lipid Prodrugs of Cidofovir and Other Phosphonates Designed for Enhanced Activity Against Smallpox and Other Orthopox Viruses.

1.0. Structure-Activity assessment of CDV analogs:

We have synthesized a series of alkoxyalkyl analogs of CDV having various chain lengths, linkers and recently a series of long chain alkyl esters without a linker moiety. These compounds are generally highly active against orthopoxviruses such as vaccinia and cowpox. Their activity is a function of the number of atoms in the chains (including linker atoms). The alkoxyalkyl series have optimal chain lengths of 18 to 24 atoms. Surprisingly, the linker with the oxygen heteroatom is not a necessary feature of the molecule which was originally designed to mimie alkylglycerol-phospholipids. Straight alkyl chains showed a sharp optimal activity at 20 atoms with EC_{50} values of 1.6 and 1.5 mM against vaccinia and cowpox. This compared with EC50s of 0.5 and o.6 in the alkoxyalkyl linker series having a chain length of 20 atoms. This paper is in press (A1, 214. Keith, KA, Wan, WB, Ciesla, SL, Beadle, JR, Hostetler, KY and Kern, ER. Inhibitory activity of alkoxyalkyl and alkyl esters of cidofovir and cyclic cidofovir against orthopoxvirus replication, in vitro, Antimicrobial Agents Chemotherapy, in press, 2003.).

1.1 Structure-Activity assessments of HMPA and PMEG versus vaccinia and cowpox *in vitro*.

Brad Wan, James Beadle and Stephanie Ciesla of our laboratory, synthesized a series of alkoxyalkanol esters of (S)HPMPA and PMEG. The compound structures, abbreviations and Antiviral Research Branch numbers (ARB numbers) are shown below.

Table 1.1.1 Derivatives of (S)HPMPA and PMEG synthesized and submitted for antiviral testing:

ARB#	Compound Name	Abbreviation
89-021	phosphonomethoxyethyl-guanine	PMEG
02-258	hexadecyloxypropyl-PMEG	HDP-PMEG
03-080	octadecyloxyethyl-PMEG	ODE-PMEG
89-018	(S)-N-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine	HPMPA
02-376	hexadecyloxypropyl-(S)HPMPA	HDP-(S)HPMPA
02-551	octadecyloxyethyl-(S)HPMPA	ODE-(S)HPMPA

These compounds were tested for efficacy against cowpox and vaccinia virus in infected HFF cells. The hexadecyloxypropyl- and octadecyloxyethyl- esters of HPMPA and PMEG were substantially more active against vaccinia and cowpox than the unmodified nucleotides, 60 to 540 fold. Their toxicity was also greater but selectivity was quite good , selectivity index of 150 to 970. These compounds seem to be excellent candidates for back up compounds for HDP-CDV and ODE-CDV, should problems be encountered in the development. The ODE-PMEG was more active than HDP-PMEG. Both analogs of HPMPA were equally active.

Table 1.1.2. Efficacy and Cytotoxicity of Ether Lipid Esters of (S)-HPMPA and PMEG

			Vaccinia Copenhagen		Cowpox Brighton	
Name	Abbreviation	CC_{50} (μM)[a]	EC_{50} (μM)[a]	SI[b]	EC_{50} (μM)[a]	SI[b]
HPMPA	(S)-HPMPA	>289 ± 38	2.7 ± 2.4	>107	4.0 ± 3.8	>72
Hexadecyloxypropyl-	HDP-(S)-HPMPA	9.7 ± 6.2	0.01 ± 0.004	970	0.02 ± 0.006	485
Octadecyloxyethyl-	ODE-(S)-HPMPA	1.5 ± 0.4	0.01 ± 0.003	150	0.02 ± 0.02	75
PMEG	PMEG	>244 ± 137	5.4 ± 2.5	>45	11 ± 1.6	>22
Hexadecyloxypropyl-	HDP-PMEG	17 ± 20	0.09 ± 0.01	189	0.1 ± 0.03	170
Octadecyloxyethyl-	ODE-PMEG	1.9 ± 1.2	0.01 ± 0.004	190	0.03 ± 0.001	63

a. Values are the mean of 2 or more assays ± standard deviation
b. .Selectivity Index (SI) = CC_{50}/EC_{50}
c.

1.1.3 Activity of HPMPA and PMEG analogs against smallpox and monkeypox.

In collaboration with Dr. John Huggins of USAMRIID we tested the HDP-PMEG and HDP-(S)HPMPA against variola major (Bangladesh strain of smallpox) or against monkeypox and the results are shown below. Both compounds were highly active

Table 1.1.4 Antiviral Activity of Novel Ether Lipid Backup Compounds against Vaccinia, Cowpox, Variola (Bangladesh) or Monkeypox Viruses *in vitro

	50% inhibitory conc,μM[1]	
Compound	Variola/BSH	Monkeypox
HDP-PMEG	<0.05	<0.05
HDP-(S)HPMPA	<0.05	<0.05

** Data of Dr.John Huggins & coworkers (V/BSH,MPX)*
[1] Neutral red reduction in Vero 76 cells

We have not received data for the EC_{50} endpoints for these compounds to date.

1.1.5. Activity of CDV analogs against IL-4 Modifed Mousepox (Ectromelia):

Previous experiments by Australian researchers indicated that the IL-4+ strain could circumvent immunization in mice, raising fears that this maneuver, if applied to smallpox, could circumvent vaccination. It is therefore of substantial interest to explore the effect of HDP-CDV and related compounds on the IL-4+ strains of ectromelia. Dr. R. Mark Buller of St. Louis University reported the

following data in Ectromelia (mousepox) and IL4 positive Ectromelia using HDP-CDV and other analogs.

Using a CV-1 cell plaque reduction assay with either ECTV-wt or an ECTV-IL-4+ recombinant virus as the indicator virus, Dr. Buller has shown that HDP-, ODE-, OLP- and OLE-CDV are highly active against both wild type and IL-4 modified ectromelia (2). He will also test the new HPMPA and PMEG analogs shown above. The compounds were sent to him in December, 2003. .

Specific Goal #2: To Assess the Oral Bioavailability, Pharmacokinetics and Toxicity of the Optimized Prodrugs of Cidofovir in Rodents, *in vivo.*

To measure the oral bioavailablilty, plasma and tissue pharmacokinetics of the alkyloxyalkanol derivatives of CDV, we contracted with Moravek Biochemicals of Brea, CA to synthesize the following compounds each having a stable radiolabel in the pyrimidine ring of cytosine:

1. 1-O-Hexadecyloxypropyl-[2-^{14}C]cidofovir
2. 1-O-Octadecyloxyethyl-[2-^{14}C]cidofovir
3. 1-O-Oleyloxypropyl-[2-^{14}C]cidofovir

Our laboratory provided them with the nonradioactive alkyloxyalkyl bromides for the reactions and Dr. Moravek followed our method to couple the unlabeled ether lipid analogs to cyclic cidofovir to give the cyclic cidofovir equivalents. We then opened the ring with sodium hydroxide and carried out *in vivo* experiments as follows. All of the compounds have been received.

2.1 Oral Bioavailability and Plasma and Tissue Pharmacokinetic Measurements with CDV Ether Lipid Conjugates:

We administered 10 mg/kg of HDP-, ODE- and OLP-[2-^{14}C]CDV orally, subcutaneously and intraperitoneally to mice and sampled plasma at 1,3,6,12,24,48 and 72 hours. The results are shown below and have been published in Antiviral Research (3):

2.1.1 Table of Oral and SC Pharmacokinetic Data:

Drug	T½	Cmax	AUCoral	AUCsc	%Oral Bioavail.
HDP-CDV	14.9	2.37	53.2	60.5	88
ODE-CDV	13.9	1.37	41.4	44.5	93
OLP-CDV	9.9	3.40	117.7	120.6	97

OLP-[^{14}C]CDV showed higher peak values of 2.9 to 3.1 μM than seen with oral HDP-CDV. Using the formula for oral bioavailability, [AUC(parenteral)/AUC(oral)] x 100, and using the area under curve from zero to 72 hours, the average oral bioavailability is calculated to be 93% for HDP-[^{14}C]CDV and 88 and 97% for ODE- and OLP-CDV, respectively. The oral bioavailability of unmodified cidofovir is <6% according to literature values reported previously by investigators from Gilead Sciences. During the 03 year, we will do oral pharmacokinetic and bioavailability studies with 1-O-octadecyloxyethyl-[^{14}C](S)HPMPA by the methods shown above.

2.2. Levels of Drug in Key Tissues Following Oral Administration of HDP-[^{14}C]CDV and ODE-[^{14}C]CDV and OLP-[^{14}C]CDV.

Since the kidney toxicity is the dose limiting toxicity for parenteral cidofovir (Vistide®), we treated mice with oral HDP-[^{14}C]CDV , ODE-[^{14}C]CDV or intraperitoneal [2-^{14}C]CDV at equimolar doses. We

removed kidney at various times and determined the amount of radioactive drug present per gram of tissue. The area under curve in kidney for for intraperitoneal CDV was 950 nmol.gm.hr versus 150 nmol.gm.hrs HDP-CDV or ODE-CDV given orally at a roughly equivalent molar dose. Thus, 6 times more i.p. CDV goes to kidney with than with oral HDP-CDV or ODE-CDV.

We carried out similar measurements in lung because of the highly important role of poxviruses in producing pneumonitis. High levels of drug in lung would be beneficial in reducing viral titers in lung as much as possible. As shown above, HDP-CDV and ODE-CDV given orally lead to much higher levels of total drug in lung over the first 72 hours than intraperitoneal CDV at a roughly equimolar dose. Elimination from lung is much slower with the ether lipid analogs, possibly due to less rapid conversion to CDV and the mono- and diphosphate analogs or to a longer half life of the intracellular cidofovir mono- and diphosphates as we showed in MRC-5 human lung cells.

In order to treat smallpox effectively, adequate drug must be delivered to the liver, lungs and spleen because these organs are key sites where the infection becomes established. As can be seen from both $AUC_{0\text{-}72}$ and C_{max} values in this publication, exposure is increased substantially in all three organs compared to CDV. The highest levels are achieved in the liver. T_{max} values in the organs for the lipid-CDV conjugates are long, but comparable to the T_{max} values calculated for the plasma. This suggests that the rate limiting step in the distribution to and uptake by the target organs is governed by the rate of uptake from the gut. These studies demonstrate clearly that the oral bioavailability of cidofovir can be increased substantially by esterification of alkoxyalkanols to CDV (4).

Specific Goal #3. To Evaluate Antiviral Activity of Selected Prodrugs of Cidofovir After Oral Administration to Animals Infected with Vaccinia Virus or Other Orthopox Viruses.

3.1 Studies with Cowpox at USAMRIID by Dr. John Huggins and coworkers

The raw data from a series of lethal cowpox challenge experiments in mice was provided by Dr. Robert O. Baker and Dr. John Huggins and is included in its entirety in the Appendix. For the purposes of the progress report I will present the most pertinent highlights from the results:

Table 3.1.1. Oral HDP-CDV in Lethal Aerosol Cowpox Infection:

Dose	Survivor/Tot	%Survival
HDP-CDV 20 mg/kg QD	9/10	90%
HDP-CDV 10 mg/kg QD	10/10	100%
HDP-CDV 5 mg/kg QD	10/10	100%
HDP-CDV 2.5 mg/kg QD	8/10	80%
HDP-CDV 1.25 mg/kg QD	7/10	70%
HDP-CDV 0.625 mg/kg QD	5/10	50%

In this study, full protection was obtained with 5 and 10 mg/kg as a single dose given daily for 5 days, starting 4 hours after infection. However, when the dose was divided into twice daily dosing, survival was slightly decreased suggesting that daily dosing is preferable. Drug toxicity was also assessed by treating animals which were uninfected with 5 daily doses of HDP-CDV: 20mg/kg (8/8); 10 mg/kg (18/18) and 5 mg/kg (18/18). All uninfected animals treated with drug survived.

Table 3.1.2. Oral HDP-CDV in Lethal Intranasal Cowpox Infection:

Dose	Survivor/Tot	%Survival
HDP-CDV 20 mg/kg QD	9/10	90%
HDP-CDV 10 mg/kg QD	10/10	100%
HDP-CDV 5 mg/kg QD	10/10	100%
HDP-CDV 2.5 mg/kg QD	8/10	80%
HDP-CDV 1.25 mg/kg QD	7/10	70%

In this study, intranasal infection followed 4 hours later by drug treatment showed full survival with 10 mg/kg HDP-CDV x5. 18 or 20 animals given 5 mg/kg/day for 5 days survived. When 10 or 5 mg/kg was given twice a day, 100% survival was noted.

Table 3.1.3. Single Oral Dose HDP-CDV in Lethal Intranasal Cowpox Infection

Dose	Day	Survivor/Tot	%Survival
HDP-CDV 140 mg/kg	-1	8/10	80%
HDP-CDV 140 mg/kg	0	1/10	10%
HDP-CDV 140 mg/kg	1	4/10	40%
HDP-CDV 140 mg/kg	2	8/10	80%
HDP-CDV 140 mg/kg	3	2/10	20%
HDP-CDV 70 mg/kg	0	7/10	70%
HDP-CDV 35 mg/kg	0	5/10	50%
HDP-CDV 18 mg/kg	0	2/10	20%
No Treatment	0	2/29	7%
HDP-CDV 140 mg/kg	Uninfected	10/10	100% [Tox control]

Single doses of oral HDP-CDV gave variable results. On day -1 (24 hrs before infection) 80% of animals survived with 140 mg/kg. However, if the same dose was given on day 0 (4 hours after infection) only 10% survived. Variable protection was noted with a single dose of 140 mg/kg on days 1, 2 and 3 after infection: 40,80,and 20%, respectively. A dose response was noted with single doses 4 hours after infection of 70, 35 and 18 mg/kg: 70, 50 and 20%. The single dose results are inferior to the 5 daily dose schedule shown above. Dr. Baker and Dr. Huggins also did some comparisons of four of the most promising alkoxylalkyl analogs of CDV given orally in lethal intranasal cowpox infection.

Table 3.1.4. Effect of Treatment Delay with Oral HDP-CDV on Mortality from Lethal Intranasal Infection with Cowpox Virus.

Dose	Rx Days	Survivor/Tot	%Survival
HDP-CDV 10 mg/kg	0-4	10/10	100%
HDP-CDV 10 mg/kg	1-5	10/10	100%
HDP-CDV 10 mg/kg	2-6	9/10	90%
HDP-CDV 10 mg/kg	3-7	6/10	60%
HDP-CDV 10 mg/kg	4-8	5/10	50%

Delaying treatment with HDP-CDV, 10 mg/kg daily for 5 days, for 1 or 2 days following infection gave 90 to 100% survival. Waiting 3 or 4 days after infection to begin treatment gave lower survival, 50 to 60%.

Table 3.1.5. Comparison of 4 Different Analogs of CDV: HDP-; ODE-; OLP- and OLE-CDV in Lethal Intranasal Cowpox Infection.

		Survivors/Total			
Dose	Rx Days	HDP-CDV	ODE-CDV	OLP-CDV	OLE-CDV
10 mg/kg	0-4	20/20	10/10	10/10	10/10
5 mg/kg	0-4	18/20	10/10	10/10	10/10
2.5 mg/kg	0-4	8/10	10/10	7/10	9/10

These studies suggest that all 4 analogs are highly effective. ODE-CDV appears to be the most effective with 100% survival at 2.5 mg/kg for 5 days. At this dose, the other compounds gave 70 to 90% survival.

In summary, 5 daily doses of HDP-CDV provided full protection at 10 or 5 mg/kg when given 4 hours to 24 hours after infection. Protection diminished if treatment was delayed for 3 or 4 days but 50% or more survived even with long treatment delays. ODE-CDV appeared to be slightly more effective than HDP-CDV but the difference may not be statistically significant. Oleyloxypropyl or oleyloxyethyl analogs were also highly effective. (John Huggins et al, unpublished, 2002-2003).

3.2 Studies with Cowpox and Vaccinia by Dr. Earl R. Kern and coworkers, University of Alabama, Birmingham.

Our studies with 4 lead compounds on oral treatment of cowpox and vaccinia in vito are in press. The abstract of this paper follows:

Four newly synthesized alkoxyalkyl esters of cidofovir (CDV): hexadecyloxypropyl-CDV (HDP-CDV), octadecyloxyethyl-CDV (ODE-CDV), oleyloxyproplyl-CDV (OLP-CDV) and oleyloxyethyl-CDV (OLE-CDV), were found to have enhanced activities against vaccinia and cowpox viruses *in vitro.* The compounds were administered orally and evaluated for efficacy against lethal cowpox virus (CV) or vaccinia virus (VV) infections in mice. HDP-CDV, ODE-CDV or OLE-CDV was effective at preventing mortality to CV when treatments were initiated 24 h post viral inoculation, but only HDP-CDV and ODE-CDV maintained efficacy when treatments were initiated as late as 72 h. Oral pretreatment with HDP-CDV or ODE-CDV was also effective when given 5, 3, or 1 day prior to virus inoculation with CV, even when administered as a single dose. Both HDP-CDV or ODE-CDV were also effective against VV infections when administered orally 24 or 48 h after infection. In animals treated with HDP-CDV or ODE-CDV, virus titers in liver, spleen and kidney were reduced 3-7 $\log_{10}$ for both CV and VV. In contrast, virus replication in lung was not significantly reduced. These data indicate that HDP-CDV or ODE-CDV given orally is as effective as parenteral CDV for the treatment of experimental CV and VV infections and suggests that the compounds may be useful for treatment of orthopoxvirus infection in humans. This paper is in press in Antimicrobial Agents and Chemotherapy (4) and a copy is in the Appendix.

3.3 Studies with Ectromelia virus (Mousepox) by Dr. R. Mark Buller, St. Louis University

We have completed initial studies of oral HDP-, ODE-, OLP- and OLE-CDV in the lethal aerosol challenge model with ectromelia virus (mousepox). The paper is in press in Virology (2) and a copy is included in the Appendix. The abstract follows:

Cidofovir (CDV) is a highly effective inhibitor of orthopoxvirus replication, and may be used intravenously to treat smallpox or complications arising from the smallpox vaccine under an investigational new drug application (IND). However, CDV is absorbed poorly following oral administration and is inactive orally. To improve the bioavailability of CDV, alkoxyalkanol esters of CDV were synthesized and were found >100 fold more active than unmodified CDV against cowpox, vaccinia, and variola virus replication. The ether lipid analogs of CDV have high oral bioavailablity in mice. In this study, we compared the oral activity of CDV with the hexadecyloxpropyl-, octadecyloxyethyl-, oleyloxypropyl- and oleyloxyethyl- esters of CDV in a lethal, aerosol ectromelia virus challenge model in A/NCR mice. Octadecyloxyethyl-CDV appeared to be the most potent CDV analog as a dose regimen of 5mg/kg completely blocked virus replication in spleen and liver, and protected 100 % of A/NCR mice, while oral, unmodified CDV was inactive. These results suggest this family of compounds deserves further evaluation as poxvirus antivirals.

3.4 Studies with Cowpox and Vaccinia Viruses by Dr. Don Smee, State University of Utah

We have completed initial studies of lethal vaccinia virus infection treated with a single oral dose of HDP-CDV in collaboration with Dr. Don Smee of the State University of Utah. This paper is in press in the International Journal of Antimicrobial Chemotherapy (5) and a copy is included in the Appendix. The abstract from this paper follows:

Intranasal infection of BALB/c mice with the IHD strain of vaccinia virus was found to cause pneumonia, profound weight loss, and death. Cidofovir, hexadecyloxypropyl-cidofovir (HDP-CDV), the diacetate ester prodrug of 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (HOE961) and ribavirin were used to treat the infections starting 24 h after virus exposure. Single intraperitoneal (i.p.) cidofovir treatments of 100 and 30 mg/kg led to 90-100% survival compared to no survivors in the placebo group, whereas a 10 mg/kg dose was ineffective. The 100 mg/kg treatment reduced lung and snout virus titers on day 3 of the infection by 20- and 8-fold, respectively. Mean arterial oxygen saturation levels in these two cidofovir treatment groups were significantly higher than placebo on days 4 through 6 of the infection, indicating an improvement in lung function. Effects of cidofovir on viral pathogenesis were studied on days 1, 3, and 5 of the infection, and demonstrated statistically significant reductions in lung consolidation scores, lung weights, lung virus titers, and snout virus titers on days 3 and 5. Cidofovir treatment also reduced virus titers in other tissues and body fluid, including blood, brain, heart, liver, salivary gland, and spleen. HDP-CDV was given orally at 100, 50 and 25 mg/kg one time only, resulting in 80-100% survival. Lower daily oral doses of 10 and 5 mg/kg/day given for 5 days protected only 30% of animals from death. Oral doses (100, 50, and 25 mg/kg/day) of HOE961 for 5 days protected all animals, whereas equivalent oral doses of ribavirin were completely ineffective. The rapidity of recovery from weight loss during the infection was a function of dose of compound administered. These data indicate the utility of parenteral cidofovir, oral HDP-CDV and oral HOE961 in treating severe respiratory infections caused by this virus.

REFERENCES

1. Keith, KA, Wan, WB, Ciesla, SL, Beadle, JR, Hostetler, KY and Kern, ER. Inhibitory activity of alkoxyalkyl and alkyl esters of cidofovir and cyclic cidofovir against orthopoxvirus replication, in vitro, Antimicrobial Agents Chemotherapy, in press, 2003.

2. Buller, R.M, Owens, G, Schriewer, J, Melman, L, Beadle JR and Hostetler, KY, Efficacy of oral active ether lipid analogs of cidofovir in a lethal mousepox model, Virology, in press, 2003.

3. Ciesla, S.L., Trahan, J., Winegarden, K.L., Aldern, K.A., Painter, G.R. and Hostetler, K.Y., Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney, Antiviral Research 59: 163-171, 2003.

4. Quenelle, D.C., D.J. Collins, K.Y. Hostetler, J.R. Beadle, W.B. Wan and E.R. Kern, Oral treatment of cowpox and vaccinia infections in mice with ether lipid esters of cidofovir, Antimicrobial Agents Chemotherapy, in press, 2003.

5. Smee, D.F., M.-H. Wong, K.W. Bailey, J.R. Beadle, K.Y. Hosteler, and R.W. Sidwell, Effects of highly active antiviral substances on lethal vaccinia virus (IDH strain) respiratory infections in mice, International J. Antimicrobial Chemotherapy, in press, 2003.

KEY RESEARCH ACCOMPLISHMENTS: 02 YEAR

- Synthesis of >30 highly active esters of cyclic cidofovir and cidofovir which are more active than unmodified CDV against variola, monkeypox, cowpox, ectromelia (mousepox) and vaccinia viruses in vitro.

- Discovery of a series of non-linker containing series of straight chain alkyl esters of cidofovir which approach the antiviral activity and selectivity of alkoxyalkyl esters of cidofovir against poxviruses.

- Publication of the oral pharmacokinetics of HDP-, ODE-, OLP-CDV in mice using 14C-labeled drug. 88 to 97% oral bioavailability demonstrated.

- Demonstration that oral HDP-[2-^{14}C]CDV given orally does not concentrate in the mouse kidney. Kidney toxicity is dose-limiting for intravenous cidofovir.

- Completed scale up synthesis of HDP-CDV (40 grams), ODE-CDV (40 gm) and characterization of the drugs by mass spectroscopy and NMR and HPLC. These compounds were used in animal efficacy studies in various model infections and have been supplied to Chimerix Inc., the NIAID contractor charged with the clinical development of these compounds.

- Oral HDP-CDV shown to provide complete protection from lethal cowpox challenge at 10 and 5 mg/kg day x 5 days by USAMRIID investigators (Huggins) and by Earl Kern and coworkers.

- Oral ODE-CDV shown to have protective effects in lethal cowpox challenge even after treatment delays of up to 48 hours. 72 hour treatment delays provide less protection. (Huggins, Kern)
- Oral HDP-CDV, 10 mg/kg, provides complete protection in lethal Ectromelia virus challenge in mice (mousepox) and ODE-CDV at 5 mg/kg provided complete protection from ectromelia. Viral titers decreased to undetectable levels in liver and spleen with lung levels generally decreased several logs by these treatments (Buller).

- Discovered two new classes of analogs of (S)HPMPA and PMEG which are 6 to 80 times more active than HDP-CDV against poxviruses including cowpox, vaccinia, and variola. These compounds will be evaluated for oral bioavailability and oral activity in lethal models of poxvirus disease in mice in the 03 year.

- 7 papers published or in press

- 9 abstracts published during the 02 year related to our work.

REPORTABLE OUTCOMES: 02 YEAR

A. Abstracts presented at National or International Meetings:

1. Karl Y. Hostetler, "Design and Development of Drugs for Smallpox", Biodefense Research, Technologies and Applications: November 5, 2002, Hilton McLean Tysons Corner, McLean, VA.

2. E. R. Kern, K. A. Keith, D. C. Quenelle, D. J. Collins, B. P. Herrod, J. R. Beadle, W. B. Wan, K. Y. Hostetler; Orally Active Alkoxyalkyl Cidofovir Prodrugs for Treatment of Orthopoxvirus Infections, ASM Biodefense Research Conference, March 9-12, 2003, Baltimore, Maryland.

3. R.M.L. Buller, G. Owens, J. Schriewer, J.R. Beadle and K.Y. Hostetler, Effect of oral ether lipid analogs of cidofovir on mortality and tissue viral titers in a lethal ectromelia virus challenge model, Proc. 16th International Conference on Antiviral Research, #131, Savannah, GA, April 2003.

4. Mucker, E.M., R.O. Baker, J.R. Beadle, J. Shamblin, D. Kefauver, S.H. Zwiers, M. Martinez, S. Ciesla, J. Trahan, K.Y. Hostetler and J.W. Huggins, Orally bioavailable alkoxyalkyl esters of cidofovir: in vitro and in vivo efficacy against orthopox viruses, Proc. 16th International Conference on Antiviral Research, Late breaker #6, Savannah, GA, April 2003.

5. Valiaeva, N. K.A. Keith, K.A. Aldern, J.R. Beadle, E.R. Kern and K.Y. Hostetler, Effects of 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG), hexadecyloxypropyl-PMEG and octadecyloxyethyl-OPMEG on replication of HIV-1, herpesviruses and poxviruses, in vitro, Proc. 16th International Conference on Antiviral Research, #111, Savannah, GA, April 2003.

6. Aldern, K.A., J.R. Beadle, S.M. Rostami, C. Hartline, E.R. Kern and K.Y. Hostetler, Alkoxyalkyl esters of adefovir: antiviral activity against cytomegalovirus and HIV-1, in vitro, Proc. 16th International Conference on Antiviral Research, #101, Savannah, GA, April 2003.

7. Trahan, J., S.L. Ciesla, K.L. Winegarden and K.Y. Hostetler, Oral pharmacokinetics and tissue distribution of 1-O-hexadecyloxypropyl-[2-^{14}C]cyclic cidofovir in mice, Proc. 16th International Conference on Antiviral Research, #132, Savannah, GA, April 2003.

8. Ciesla, S.L., W.B. Wan, K.A. Aldern, J.R. Beadle and K.Y. Hostetler, Synthesis of alkoxyalkyl esters of (S)-HPMPA and antiviral activity against herpesviruses, in vitro, Proc. 16th International Conference on Antiviral Research, #102, Savannah, GA, April 2003.

9. Keith, K.A., W.B. Wan, S.L. Ciesla, J.R. Beadle, K.Y. Hostetler and E.R. Kern, Inhibitory effect of alkoxyalkyl esters of acyclic nucleoside phosphonates against orthopoxvirus replication, Proc. 16th International Conference on Antiviral Research, #110, Savannah, GA, April 2003.

B. Manuscripts published or submitted

1. Aldern, K.A., Ciesla, S.L., Winegarden, K.L. and Hostetler, K.Y., The increased antiviral activity of 1-O-hexadecyloxypropyl-cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism, Molecular Pharmacology, 63:678-681, 2003.

2. Ciesla, S.L., Trahan, J., Winegarden, K.L., Aldern, K.A., Painter, G.R. and Hostetler, K.Y., Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney, Antiviral Research 59: 163-171, 2003.

3. Smee, D.F., M.-H. Wong, K.W. Bailey, J.R. Beadle, K.Y. Hosteler, and R.W. Sidwell, Effects of highly active antiviral substances on lethal vaccinia virus (IDH strain) respiratory infections in mice, International J. Antimicrobial Chemotherapy, in press, 2003.

4. Quenelle, D.C., D.J. Collins, K.Y. Hostetler, J.R. Beadle, W.B. Wan and E.R. Kern, Oral treatment of cowpox and vaccinia infections in mice with ether lipid esters of cidofovir, Antimicrobial Agents Chemotherapy, in press, 2003.

5. Buller, R.M, Owens, G, Schriewer, J, Melman, L, Beadle JR and Hostetler, KY, Efficacy of oral active ether lipid analogs of cidofovir in a lethal mousepox model, Virology, in press, 2003.

6. Keith, KA, Wan, WB, Ciesla, SL, Beadle, JR, Hostetler, KY and Kern, ER. Inhibitory activity of alkoxyalkyl and alkyl esters of cidofovir and cyclic cidofovir against orthopoxvirus replication, in vitro, Antimicrobial Agents Chemotherapy, in press, 2003.

7. Bidanset, D.J., Beadle, J.R., Wan, W.B., Hostetler, K.Y. and Kern E.R., Oral activity of ether lipid prodrugs of cidofovir against experimental human cytomegalovirus infections, J. Virology, in press, 2003.

C. Presentations Pending

1. Hostetler, Karl, *Development of Drugs for Prevention and Treatment of Smallpox,* Keystone Biodefense Conference, January 9, 2004, Keystone, CO.

2. Hostetler, Karl, *Development of Drugs for Prevention and Treatment of Smallpox.* Gordon Research Conference, January 17, 2004, Santa Barbara, CA.

D. Awards

1. "Oral Smallpox Drug", Winner, *Popular Science* Magazine's 2002 Best of Whats New Award., December issue of *Popular Science*.

2. San Diego City Club Citation for Biodefense Work in 2002, University of California, San Diego & Hostetler laboratory contribution: *Development of oral treatments for smallpox.*

CONCLUSIONS:

In the second year of work under this Army grant, we have continued to evaluate structure-activity relationships in esters of cidofovir and other antiviral phosphonates. We have synthesized over 30 active antiviral compound active against variola, vaccinia, monkeypox, cowpox and mousepox. Most of these compounds are substantially more active and selective than cidofovir. We have also discovered a new series of active compounds which have no linker moiety. The most active of these is a 20 carbon straight alkyl chain (docosanol) esterified to CDV. Our lead compounds, 1-O-hexadecyloxypropyl-cidofovir (HDP-CDV) and 1-O-octadecyloxypropyl-cidofovir (ODE-CDV), have excellent orally bioavailability and prevent death from lethal cowpox and mousepox viral challenge. Four papers on oral activity or our drugs are currently in press in peer reviewed journals. We have also identified two new classes of highly active anti-poxvirus drugs NOT based on cidofovir, (S)HPMPA and PMEG analogs using our design are also highly active against variola and other poxviruses. We have provided 40 grams of HDP-CDV and ODE-CDV to the NIAID contractor charged with the clinical development of these agents for dose ranging toxicology studies.

In the coming 03 year of the project we will continue to support our collaborators at USAMRIID by providing new analogs of (S)HPMPA and PMEG for in vitro and in vivo animal studies against orthopoxviruses. We will also intensify our research on the two new compounds to determine oral bioavailability and activity in various animal models of poxvirus disease. We do not believe that any changes in general direction or activities is necessary beyond those described in the original proposal.

With regard to possible medical products, we believe that HDP-CDV or ODE-CDV represent excellent candidates drugs for oral prevention or treatment of smallpox. The best of these two drugs could be developed to IND filing and human safety testing in order to provide a therapy/preventative for immunosuppressed Americans or Americans with eczema or atopic dermatitis who cannot be safely vaccinated. Final proof of concept would require successful testing in an FDA acceptable primate model of smallpox like that currently being developed by Dr. Peter Jahrling and Dr. John Huggins of USAMRIID.

APPENDIX TO

"Development of Potent Orally Active Agents for Prevention and Treatment of Smallpox"

DAMD 17-01-2-0071
Karl Y. Hostetler, M.D.
Principal Investigator

December 17, 2003

Manuscripts published and in press

1. Aldern, K.A., Ciesla, S.L., Winegarden, K.L. and Hostetler, K.Y., The increased antiviral activity of 1-O-hexadecyloxypropyl-cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism, Molecular Pharmacology, 63:678-681, 2003.

2. Ciesla, S.L., Trahan, J., Winegarden, K.L., Aldern, K.A., Painter, G.R. and Hostetler, K.Y., Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney, Antiviral Research 59: 163-171, 2003.

3. Smee, D.F., M.-H. Wong, K.W. Bailey, J.R. Beadle, K.Y. Hosteler, and R.W. Sidwell, Effects of highly active antiviral substances on lethal vaccinia virus (IDH strain) respiratory infections in mice, International J. Antimicrobial Chemotherapy, in press, 2003.

4. Quenelle, D.C., D.J. Collins, K.Y. Hostetler, J.R. Beadle, W.B. Wan and E.R. Kern, Oral treatment of cowpox and vaccinia infections in mice with ether lipid esters of cidofovir, Antimicrobial Agents Chemotherapy, in press, 2003.

5. Buller, R.M, Owens, G, Schriewer, J, Melman, L, Beadle JR and Hostetler, KY, Efficacy of oral active ether lipid analogs of cidofovir in a lethal mousepox model, Virology, in press, 2003.

6. Keith, KA, Wan, WB, Ciesla, SL, Beadle, JR, Hostetler, KY and Kern, ER. Inhibitory activity of alkoxyalkyl and alkyl esters of cidofovir and cyclic cidofovir against orthopoxvirus replication, in vitro, Antimicrobial Agents Chemotherapy, in press, 2003.

7. Bidanset, D.J., Beadle, J.R., Wan, W.B., Hostetler, K.Y. and Kern E.R., Oral activity of ether lipid prodrugs of cidofovir against experimental human cytomegalovirus infections, J. Virology, in press, 2003.

0026-895X/03/6303-678–681$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 63:678–681, 2003

Vol. 63, No. 3
2187/1044993
Printed in U.S.A.

Increased Antiviral Activity of 1-*O*-Hexadecyloxypropyl-[2-^{14}C]cidofovir in MRC-5 Human Lung Fibroblasts Is Explained by Unique Cellular Uptake and Metabolism

KATHY A. ALDERN, STEPHANIE L. CIESLA, KRISTINE L. WINEGARDEN, and KARL Y. HOSTETLER

Department of Medicine, San Diego VA Healthcare System and the University of California, San Diego, La Jolla, California

Received October 1, 2002; accepted November 26, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Recently, there has been renewed interest in finding orally active drugs against smallpox. Cidofovir (CDV) given by parenteral injection has been shown to protect against lethal poxvirus infection. We have been interested in the synthesis and evaluation of orally active derivatives of CDV. Previous studies showed that the CDV and cyclic cidofovir (cCDV) analogs 1-*O*-hexa-decyloxypropyl-CDV (HDP-CDV) and 1-*O*-hexadecyloxypropyl-cCDV (HDP-cCDV), show >100-fold increases in antiviral activity versus the unmodified nucleosides against cells infected with orthopoxviruses, cowpox, and vaccinia virus. In contrast to CDV, HDP-CDV is orally bioavailable and has been reported to be orally active in lethal cowpox virus infection in mice. To assess the metabolic basis for the increased antiviral activity of HDP-CDV in vitro, we studied the cellular uptake and anabolic metabolism of ^{14}C-labeled CDV, cCDV, and their alkoxyalkanol esters HDP-CDV and HDP-cCDV. HDP-CDV and HDP-cCDV were taken up rapidly by MRC-5 human lung fibroblasts in vitro, but uptake of CDV and cCDV was much slower. Analysis of cellular metabolites showed that levels of cidofovir diphosphate (CDV-DP), the active antiviral compound, were >100 times greater with HDP-CDV than levels observed with CDV. When cells were exposed to HDP-CDV, the intracellular half-life of CDV-DP was 10 days versus 2.7 days reported when cells are exposed to CDV. HDP-CDV seems to circumvent poor cellular uptake by rapid association with cellular membrane phospholipids, whereas CDV uptake proceeds via the slow process of fluid endocytosis.

Cidofovir (1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl] cytosine; CDV) is an acyclic phosphonate analog of cytosine that has been shown to have activity against all double-stranded DNA viruses studied to date, including herpes group viruses, orthopoxviruses, parapoxviruses, adenoviruses, and papovaviruses (De Clercq et al., 1987; Snoeck et al., 1988; De Clercq, 1997). CDV (Vistide; Gilead Sciences, Foster City, CA) is approved as an intravenous treatment for cytomegalovirus retinitis in AIDS patients but has dose-limiting renal side effects (Lea and Bryson, 1996; Plosker and Noble, 1999). CDV given intravenously protects mice against lethal vaccinia or cowpox virus infection (Neyts and De Clercq, 1993; Bray et al., 2000; Smee et al., 2000). Topical cidofovir has also been reported to have activity against mollusca contagiosum (Zabawski, 2000). Intralesional CDV has been used to treat laryngeal papillomatosis (Snoeck et al., 1998; Stragier et al., 2002).

It would be useful to have highly active antiviral analogs of CDV that are less toxic and orally bioavailable. Our laboratory has developed a strategy to enhance absorption of poorly absorbed nucleotides, such as acyclovir monophosphate and ganciclovir monophosphate, by attaching certain ether lipid residues, such as 1-*O*-hexadecylpropanediol (Hostetler et al., 1997, 2000, 2001; Beadle et al., 2000). As part of this program, we synthesized 1-*O*-hexadecyloxypropyl-CDV (HDP-CDV) and tested it against MRC-5 human lung fibroblasts infected with cytomegaloviruses and herpes simplex viruses, type 1 and type 2. HDP-CDV exhibited multiple log increases in antiviral activity in vitro against CMV and HSV-1 compared with CDV (Beadle et al., 2002) (Table 1). HDP-CDV was also active against various ganciclovir-resistant CMV isolates. Multiple log enhancement of antiviral activity was also noted against various strains of cowpox and vaccinia virus infected cells in vitro (Kern et al., 2002) (Table 1) and against variola virus-infected cells in vitro (J. W. Huggins, personal communication).

This work was funded in part by National Eye Institute grant EY11834, National Institute of Allergy and Infectious Disease grant AI29164, and Department of the Army grant DAMD 17-01-2-007. The U.S. Army Medical Research Acquisition Activity, Fort Detrick, MD, is the awarding acquisition office.

The content of this article does not necessarily reflect the position or policy of the government, and no official endorsement should be inferred.

K.Y.H. is a consultant to Chimerix, Inc., the licensee of HDP-CDV.

ABBREVIATIONS: CDV, cidofovir; HDP, 1-*O*-hexadecyloxypropyl; HSV, herpes simplex virus; CMV, cytomegalovirus; cCDV, cyclic cidofovir; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; CDV-MP, cidofovir monophosphate; CDV-DP, cidofovir diphosphate; HCMV, human cytomegalovirus.

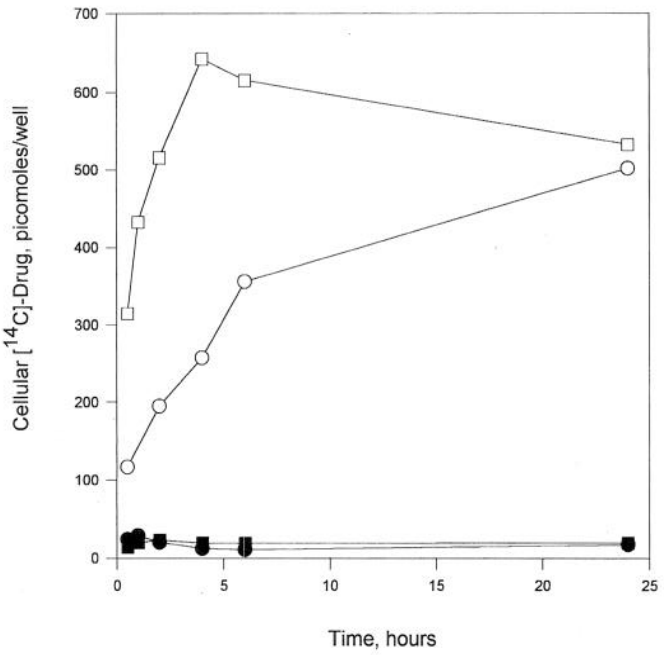


Fig. 1. Uptake of ^{14}C-labeled drugs by MRC-5 human lung fibroblasts. Data are the average of two determinations. ●, CDV; ■, cyclic CDV; ○, HDP-CDV; □, HDP-cyclic CDV.

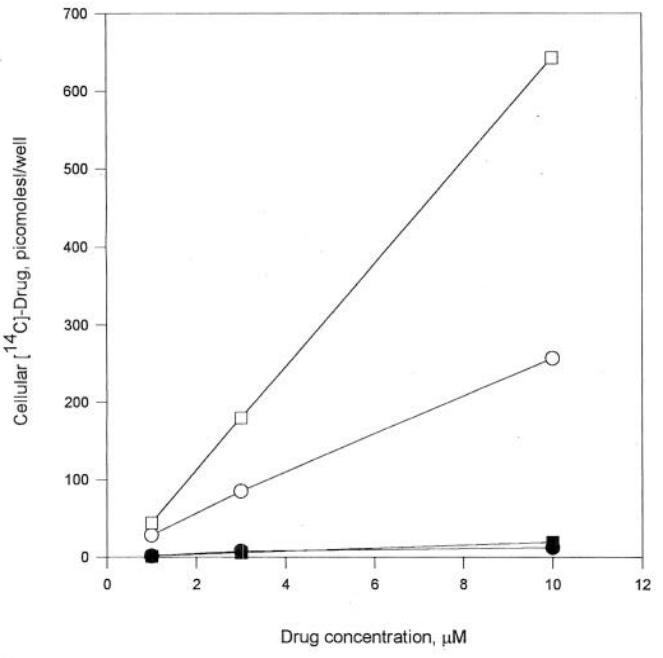


Fig. 2. Effect of concentration of cellular uptake of ^{14}C-labeled drugs. Cells were exposed to drugs for 4 h and analyzed for drug content. ●, CDV; ■, cyclic CDV; ○, HDP-CDV; □, HDP-cyclic CDV.

^{14}C]CDV for 24 h. Then the radioactive drug was washed away with PBS and the medium was replaced with drug-free growth medium and incubation continued for 2 to 10 days. Cell metabolites were analyzed by HPLC at 0, 2, 4, 6, 8, and 10 days after removal of the drug. Cell extracts were prepared by freezing and thawing in water, and the membrane fraction was isolated by centrifugation. The membrane fraction contained unmetabolized HDP-[2-^{14}C]CDV, which represented 2084 pmol/flask at time 0. The water-soluble metabolites consisted of 460 pmol/flask CDV, 45 pmol/flask CDV-MP, and 83 pmol/flask CDV-DP at zero time (Fig. 3). Two days after the washout of HDP-[2-^{14}C]CDV from the flask, membrane levels of HDP-[2-^{14}C]CDV declined by 52%, whereas the water-soluble metabolites [2-^{14}C]CDV, [2-^{14}C]CDV-MP, and [2-^{14}C]CDV-DP increased by 58, 102, and 64%, reaching peak levels of 722, 74, and 166 pmol/flask, respectively. Thereafter, CDV, CDV-MP, and CDV-DP declined gradually to 267, 24, and 83 pmol/flask at 10 days. The $T_{1/2}$ values for HDP-CDV, CDV, CDV-MP, and CDV-DP were estimated to be 2, 8, 7, and 10 days, respectively (Fig. 3).

Discussion

Cellular uptake of CDV is slow and has been shown to occur by fluid phase endocytosis (Connelly et al., 1993). Covalent addition of the 1-*O*-hexadecyloxypropyl ester to the phosphonate of CDV results in remarkable increases in the antiviral activity of HDP-CDV versus CDV against HCMV and HSV (Beadle et al., 2002) and against vaccinia virus and cowpox viruses (Kern et al., 2002) (Table 1). The present study indicates that this is caused, at least in part, by increased cell penetration of HDP-CDV relative to CDV. Furthermore, the intracellular levels of the active antiviral metabolite, CDV-DP, formed after intracellular cleavage of HDP-CDV by phospholipase C-like enzymes and phosphorylation by cellular kinases, is more than two logs greater than the levels observed with equimolar concentrations of CDV. The intracellular half-life of CDV-DP is approximately 10 days in cells exposed to HDP-CDV versus a reported half-life in cells exposed to CDV of 17 h (Ho et al., 1992) or 1 to 2.7 days in Vero cells, where a biphasic decay of CDV-DP was observed (Aduma et al., 1995). The ratios of CDV to CDV-MP and CDV-DP observed when cells were exposed to HDP-CDV were generally similar to that seen with CDV alone, as reported by Ho and coworkers (1992) and Aduma et al. (1995) (i.e., CDV $\gg$ CDV-DP $>$ CDV-MP). Surprisingly, we did not observe conversion of CDV to CDV diphosphate choline in these experiments, in contrast to prior reports (Ho et al., 1992; Aduma et al., 1995). An important cause of the 10-day $T_{1/2}$ value observed for CDV-DP after exposure of cells to

TABLE 2

Comparison of metabolite levels found in MRC-5 cells after exposure to [2-^{14}C]CDV (10 μM) or HDP-[2-^{14}C]CDV (10 μM)

Times are the time of exposure to radioactive CDV or HDP-CDV. Each data point represents an analysis of the cells from a single T-75 flask.

Metabolite	CDV			HDP-CDV		
	6 h	24 h	48 h	6 h	24 h	48 h
	pmol/flask					
HPMPU	4.8	6.9	4.2	8.8	27.3	36.0
CDV	146.4	273.8	129.3	166.7	697.4	702.0
CDV-MP	3.9	1.0	1.2	11.8	63.2	71.4
CDV-DP	6.3	1.3	1.8	11.2	132.6	184.4

HPMPU, (*S*)-1-[3-hydroxy-2-(phosphonoylmethoxy)propyl]uridine.

Reprinted from

Antiviral Research

Antiviral Research 59 (2003) 163–171

Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney

Stephanie L. Ciesla[a], Julissa Trahan[a], W. Brad Wan[a], James R. Beadle[a], Kathy A. Aldern[a], George R. Painter[b], Karl Y. Hostetler[a,*]

[a] *Department of Medicine (0676), San Diego VA Healthcare System, University of California, San Diego, 305 Stein Clinical Research Building, 9500 Gilman Drive, La Jolla, CA 92093-0676, USA*

[b] *Chimerix Inc., 5015 Southpark Drive, Suite 240, Durham, NC 27713, USA*

ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 59 (2003) 163–171
Antiviral Research
www.elsevier.com/locate/antiviral

Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney

Stephanie L. Ciesla[a], Julissa Trahan[a], W. Brad Wan[a], James R. Beadle[a], Kathy A. Aldern[a], George R. Painter[b], Karl Y. Hostetler[a,*]

[a] *Department of Medicine (0676), San Diego VA Healthcare System, University of California, San Diego, 305 Stein Clinical Research Building, 9500 Gilman Drive, La Jolla, CA 92093-0676, USA*
[b] *Chimerix Inc., 5015 Southpark Drive, Suite 240, Durham, NC 27713, USA*

Received 11 February 2003; accepted 24 April 2003

Abstract

Smallpox was eradicated by vaccination in the 1970s. However, concerns have arisen about the potential use of variola virus as a biological weapon. Most of the world's population has little residual immunity because systematic vaccination against smallpox ceased in the early 1970s. Vaccination of key elements of the population against smallpox is again being considered. However, there are now large numbers of persons who cannot be safely vaccinated with the current vaccine because of AIDS, immunosuppressive drugs, and certain common skin disorders. It would be useful to have a potent orally active drug as an alternative for these persons in case of an outbreak of smallpox.

Alkoxyalkyl esters of cidofovir (CDV) have been shown to be highly active and selective against poxviruses in vitro with activities several logs greater than the activity of unmodified CDV. This is due in large part to increased cellular penetration and conversion to CDV-diphosphate, the active antiviral. In this paper, the oral pharmacokinetics of ^{14}C-labeled hexadecyloxypropyl-cidofir (HDP-CDV), octadecyloxyethyl-cidofir (ODP-CDV), and oleyloxypropyl-cidofir (OLP-CDV) are examined and oral bioavailability and tissue distribution assessed and compared with parenteral CDV. The alkoxyalkyl CDVs are highly orally bioavailable and do not concentrate in kidney, the site of the dose-limiting toxicity of CDV. Plasma and tissue drug levels are many times greater than the in vitro EC_{50s} for variola, cowpox, and vaccinia viruses. Thus, the compounds are good candidates for further development for prevention and treatment of smallpox infection and the complications of vaccination.
Published by Elsevier B.V.

Keywords: Esterification; Alkoxyalkanols; Oral bioavailability

Cidofovir (CDV, Vistide®; 1-[(S)-9-(hydroxyphosphonomethyl-propyl)cytosine]), is a potent and selective inhibitor of viral DNA synthesis after conversion by cellular kinases to the diphosphate (Snoek et al., 1988; De Clercq et al., 1987; Ho et al., 1992; Cherrington et al., 1994; Xiong et al., 1996), and is FDA approved for the treatment of cytomegalovirus retinitis in AIDS patients (Lalezari et al., 1997; Studies of Ocular Complications Authors, 1997). However, owing to poor oral bioavailability of <5% (Wachsman et al., 1996; Cundy, 1999), CDV must be administered by intravenous infusion. In addition, CDV has exhibited treatment-limiting nephrotoxicity resulting in the requirement for prehydration, slow intravenous infusion and concomitant treatment with Probenecid.

CDV is a broad spectrum antiviral that shows in vitro activity against other DNA viruses including the herpes viruses, orthopoxviruses, polyomavirus, papillomavirus, and adenoviruses (reviewed in De Clercq, 2002). CDV is also active in vivo against lethal vaccinia and cowpox challenge experiments in rodents when given by injection including the intraperitoneal, intravenous, and subcutaneous routes of administration (De Clercq and Neyts, 1993; Bray et al., 2000; Martinez et al., 2000; Smee et al., 2001; Huggins et al., 2002).

We reported recently that the in vitro activity of CDV against herpes group viruses (HCMV and HSV) and orthopoxviruses (vaccinia and cowpox) could be increased

Abbreviations: CDV, cidofovir; HDP-CDV, hexadecyloxypropyl-cidofovir; ODE-CDV, octadecyloxyethyl-cidofovir; OLP-CDV, oleyloxypropyl-cidofovir

* Corresponding author. Tel.: +1-858-552-8585x2616; fax: +1-858-534-6133.
E-mail address: khostetl@ucsd.edu (K.Y. Hostetler).

0166-3542/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/S0166-3542(03)00110-4

radioactive compounds were dried under nitrogen gas and rehydrated with sterile 0.9% saline, followed by sonication to obtain clear solutions.

1.2. Pharmacokinetic studies

Female NIH Swiss mice (Harlan–Sprague–Dawley,) at a weight range of 20–25 g were treated by the routes and dosages indicated with [2-^{14}C]CDV or HDP-, ODE-, or OLP-[2-^{14}C]CDV. At times ranging from 1 to 72 h, blood was collected in heparinized tubes and the mice were euthanised. In tissue distribution experiments, spleen, liver, kidney, and lung tissues were removed at the indicated times, weighed, and solubilized with TS-2 (Research Products International, Mt. Prospect, IL) and processed for liquid scintillation counting. The blood was centrifuged and the drug content of a 50 μl aliquot was determined by liquid scintillation counting. Plasma and organ drug content is reported as nanomol drug per milliliter (plasma) and nanomol drug per gram (organs). Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institute of Health.

1.3. Analysis of plasma

In some experiments with oral HDP-[2-^{14}C]CDV, plasma was subjected to lipid extraction to separate CDV from HDP-CDV (Folch et al., 1957). The aqueous and lipid phases were separated and the upper phase was washed twice with ideal lower phase (as defined in Folch et al., 1957). The original lower phase and the two washes were combined. Aliquots of the upper and the combined lower phases were removed for liquid scintillation counting. The lower phase was also analyzed by thin layer chromatography on silica gel G layers developed with chloroform:methanol:ammonia:water (70:58:8:8) by volume. The plates were scanned with a Radiomatic RTLC scanner (Radiomatic, Tampa, FL) and the peaks were compared with reference standards of HDP-[2-^{14}C]CDV and [2-^{14}C]CDV.

Plasma lipid extracts were also analyzed by HPLC as described previously (Aldern et al., 2003). Aliquots of the upper phases of the lipid extraction were applied to a 4.6 cm × 15 cm Partisil 10 SAX column with a SAX guard column. The column was eluted at a flow rate of 1 ml/min using a potassium phosphate buffer gradient of 20–700 mM, pH 5.8, beginning at 9 min for a duration of 20 min and a terminal hold of 5 min. Fractions were collected in 1 min/ml and FloScint IV scintillation fluid was added and the samples were analyzed by liquid scintillation counting. The lower phase from the lipid extractions were also analyzed by HPLC as follows. A small aliquot of the lower phase, 50–75 μl, was applied to a 4.6 cm × 15 cm Waters XTerra column with a 3.9 mm × 20 mm Waters XTerra guard column (Waters Corp, Milford, MA). The samples were eluted with 80% methanol at a flow rate of 0.5 ml/min for 15 min. One milliliter of fractions were collected and analyzed as described above. Radioactivity in each fraction was plotted versus time and the fractions containing HDP-CDV were determined by comparison with the retention time of an unlabeled HDP-CDV standard run under identical conditions.

2. Results

2.1. Dose dependence of plasma drug levels

HDP-[2-^{14}C]CDV was administered by oral gavage to mice at doses of 5, 10, and 20 mg/kg. For comparison, [2-^{14}C]CDV was given orally at 5.6 mg/kg (a dose which is the molar equivalent of the CDV delivered by 10 mg/kg of HDP-CDV). Plasma was obtained at times from 1 to 24 h postdose and drug levels determined as described in methods (Fig. 2). Plasma CDV levels were highest 1 h after oral dosing, 0.12 μM, and declined to 0.007 μM at 24 h. Oral administration of HDP-CDV produced substantially higher plasma levels. At 5 mg/kg, a peak value of 0.8 μM was reached at 6 h. At 10 mg/kg a plateau was observed between 3 and 12 h with a peak value of 2.37 μM at 12 h. At 20 mg/kg, delayed appearance of drug in plasma was noted relative to the results obtained with the 5 and 10 mg/kg doses. A peak level of 3.17 μM HDP-CDV occurred at 12 h, declining to 1.67 at 24 h.

Area under curve values ($AUC_{0-24\,h}$) were as follows: CDV 5.6 mg/kg, 0.81 units; HDP-CDV 5 mg/kg, 16.3 units; 10 mg/kg, 38.8 units, and 20 mg/kg, 16.5 units. At molar equivalent doses, the $AUC_{0-24\,h}$ of oral HDP-CDV in mouse plasma is 48 times greater than that of oral CDV. The $AUC_{0-24\,h}$ values for 5 and 10 mg/kg of HDP-CDV were roughly proportional to dose, but the $AUC_{0-24\,h}$ value for 20 mg/kg HDP-CDV was significantly lower than expected because much of the decline of drug in plasma occurred after 24 h and was not measured. Therefore, in the subsequent oral bioavailability experiments, plasma drug levels were measured out to 72 h.

2.2. Assessment of oral bioavailability

HDP-[2-^{14}C]CDV (10 mg/kg in normal saline) was administered to mice by the subcutaneous, intraperitoneal and oral routes and plasma was obtained at times ranging from 1 to 72 h. Similar experiments were carried out with ODE-[2-^{14}C]CDV and OLP-[2-^{14}C]CDV except that these drugs were only administered by the oral and subcutaneous routes (Fig. 3). The pharmacokinetic data derived from this data is shown in Table 1. When given by the intraperitoneal or subcutaneous routes, HDP-CDV drug levels in plasma peaked 3 or 6 h after administration at 3.1 and 2.7 μM, respectively. Oral HDP-CDV reached a plateau after 3 h with a peak of 2.4 μM at 12 h. After 12 h the oral plasma drug levels declined with a rate similar to that of the parenterally administered HDP-CDV. Comparison of the oral and parenteral

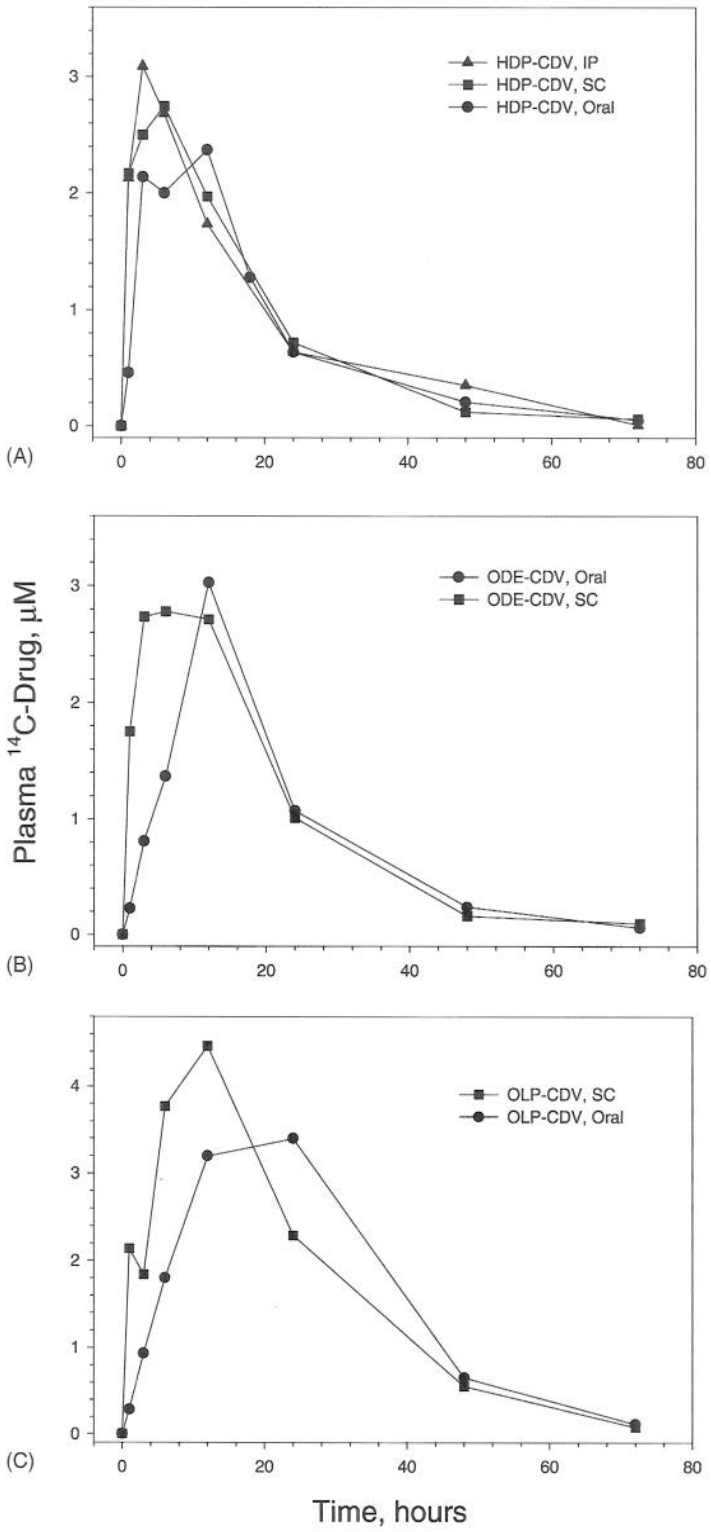


Fig. 3. Determination of the oral bioavailability of HDP-CDV, ODE-CDV, and OLP-CDV in mice. Mice were given 10 mg/kg of the indicated compounds by the orally (closed circles), subcutaneously (squares), or intraperitoneally (triangles). HDP-CDV (A), ODE-CDV (B), and OLP-CDV (C). The data represent all species of ^{14}C-labeled drug in plasma (µmol) at the indicated times following administration. The data are the average of three separate determinations. The pharmacokinetic data is summarized in Table 1.

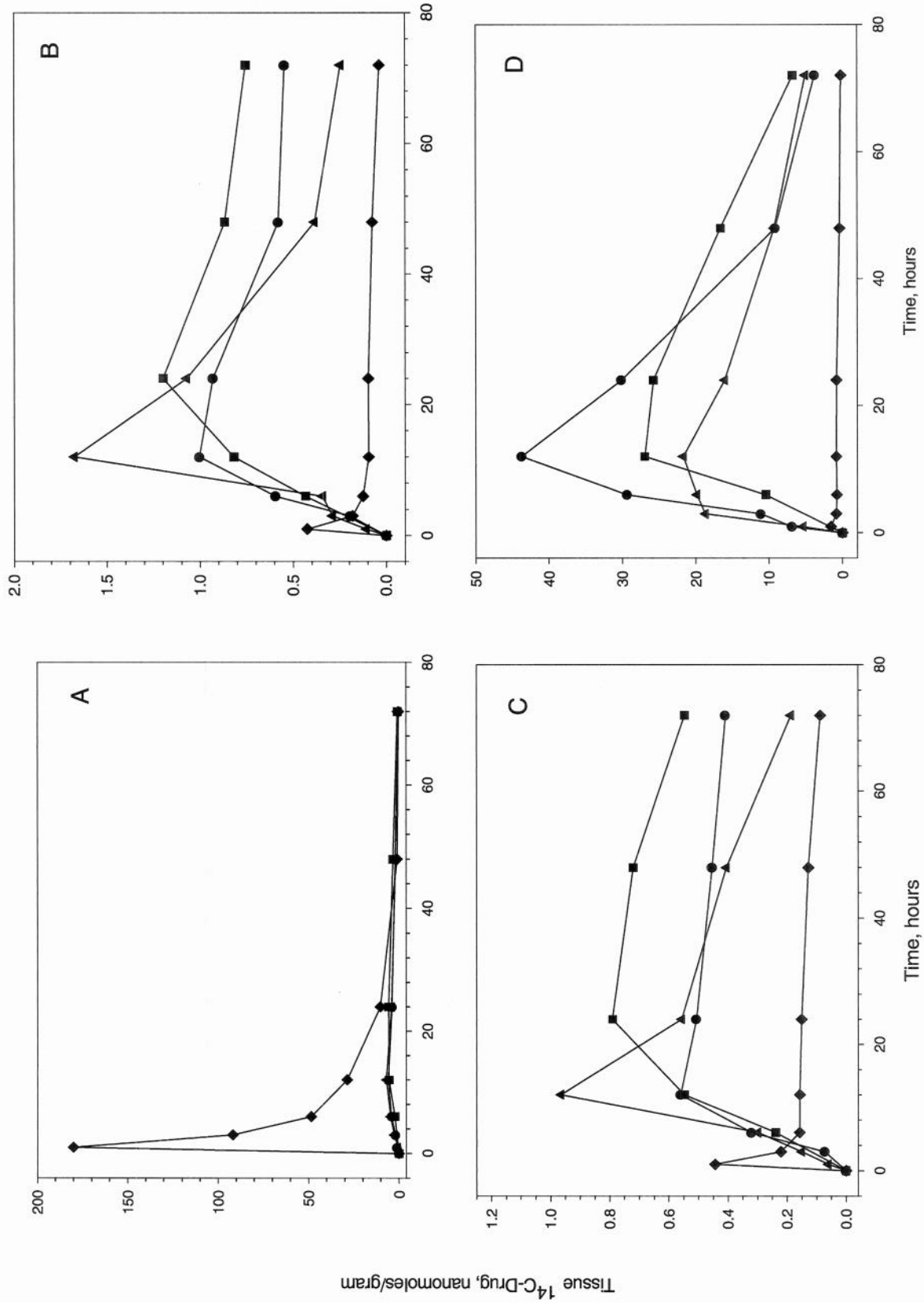


Fig. 4. Tissue levels of ^{14}C-drug following oral administration of three alkoxyalkanol analogs of CDV versus an equimolar dose of intraperitoneal CDV. Kidney (A), lung (B), spleen (C), and liver (D). Data are nanomole per grams of ^{14}C-labeled drug (all species) and represent the average of three determinations. Oral HDP-CDV, circles; oral ODE-CDV, squares; oral OLP-CDV, triangles; and intraperitoneal CDV. Alkoxyalkanol CDV doses were 10 mg/kg and the intraperitoneal dose of CDV was 5.6 mg/kg (the molar equivalent dose). Pharmacokinetic data is summarized in Table 2.

Beadle, J.R., Kini, G.D., Aldern, K.A., Gardner, M.F., Wright, K., Hostetler, K.Y., 1998. Alkylthioglycerol prodrugs of foscarnet: oral bioavailability and structure-activity studies in human cytomegalovirus, herpes virus type-1 and human immunodeficiency virus type-1 infected cells. Antivir. Chem. Chemother. 9, 33–40.

Beadle, J.R., Kini, G.D., Aldern, K.A., Gardner, M.F., Wright, K.N., Ryback, R.J., Kern, E.R., Hostetler, K.Y., 2000. Synthesis and antiviral evaluation of 1-*O*-hexadecylpropanediol-3-*P*-acyclovir: efficacy against HSV-1 infection in mice. Nucleosides Nucleotides 19, 471–480.

Beadle, J.R., Rodriquez, N., Aldern, K.A., Hartline, C., Harden, E., Kern, E.R., Hostetler, K.Y., 2002. Alkoxyalkyl esters of cidofovir and cyclic cidofovir exhibit multiple log enhancement of antiviral activity against cytomegalovirus and herpesvirus replication, in vitro. Antimicrob. Agents Chemother. 46, 2381–2386.

Bray, M., Martinez, M., Smee, D.F., Kefauver, D., Thompson, E., Huggins, J.W., 2000. Cidofovir protects mice against lethal aerosol or intranasal cowpox virus challenge. J. Infect. Dis. 181, 10–19.

Cherrington, J.M., Allen, S.J.W., McKee, B.H., Chen, M.S., 1994. Kinetic analysis of the interaction between the diphosphate of (S)-1-[3-hydroxy-2-(phosphonylmethoxyl)propyl]-cytosine, zalcitibine-TP, zidovudine-TP and FIAUTP with human DNA polymerases b and g. Biochem. Pharmacol. 48, 1986–1988.

Cundy, K.C., 1999. Clinical pharmacokinetics of the antiviral nucleotide analogs cidofovir and adefovir. Clin. Pharmacokinet. 36, 127–143.

De Clercq, E., 2002. Cidofovir in the treatment of poxvirus infections. Antivir. Res. 55, 1–13.

De Clercq, E., Neyts, J., 1993. Efficacy of (S)-1-(3-hydroxy-2-phosphonylmethoxy- propyl)cytosine for the treatment of lethal vaccinia virus infections in severe combined immune deficiency (scid) mice. J. Med. Virol. 41, 242–246.

De Clercq, E., Sakuma, T., Baba, M., Pauwels, R., Balzarini, J., Rosenberg, I., Holy, A., 1987. Antiviral activity of phosphonylmethoxyalkyl derivatives of purine and pyrimidines. Antivir. Res. 8, 261–272.

Folch, J.M., Leas, M., Sloane-Stanley, G.H., 1957. A simple procedure for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Henderson, D.A., Inglesby, T.V., Bartlett, J.G., Ascher, M.S., Eitzen, E., Jahrling, P.B., Hauer, J., Layton, M., McDade, J., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T., Russell, P.K., Tonat, K., and the Working Group on Civilian Biodefense, 1999. JAMA 281, 2127–2137.

Ho, H.T., Woods, K.L., Bronson, J.J., De Boeck, H., Martin, J.C., Hitchcock, M.J.M., 1992. Intracellular metabolism of the antiherpesvirus agent (S)-1-[3-hydroxy-2-(phosphonyl-methoxyl)propyl]cytosine. Mol. Pharmacol. 41, 197–202.

Ho, E.S., Lin, C., Mendel, D.B., Ahlor, T., 2000. Cytotoxicity of antiviral nucleosides adefovir and cidofovir is induced by the expression of human renal organic anion transporter 1. J. Am. Soc. Nephrol. 11, 383–393.

Hostetler, K.Y., Beadle, J.R., Kini, G.D., Gardner, M.F., Wright, K.N., Wu, T.H., Korba, B.A., 1997. Enhanced oral absorption and antiviral activity of 1-*O*-octadecyl-*sn*-glycero-3-phospho-acyclovir and related compounds in hepatitis B virus infection, in vitro. Biochem. Pharmacol. 53, 1815–1822.

Hostetler, K.Y., Beadle, J.R., Hornbuckle, W.E., Bellezza, C.A., Tochkov, I.A., Cote, P.J., Gerin, J.L., Korba, B.E., Tennant, B.C., 2000. Antiviral activity of oral 1-*O*-hexadecylpropanediol-3-P-ACV in woodchucks with chronic woodchuck hepatitis virus infection. Antimicrob. Agents Chemother. 44, 1964–1969.

Hostetler, K.Y., Rybak, R.J., Beadle, J.R., Hartline, C.B., Gardner, M.F., Aldern, K.A., Wright, K., Kern, E.R., 2001. In vitro and in vivo activity of 1-*O*-hexadecylpropanediol-3-phospho-ganciclovir and 1-*O*-hexadecylpropanediol-3-phospho-penciclovir in cytomegalovirus and herpes simplex virus infections. Antivir. Chem. Chemother. 11, 373–381.

Huggins, J.W., Baker, R.O., Beadle, J.R., Hostetler, K.Y., 2002. Orally active ether lipid prodrugs of cidofovir for the treatment of smallpox. Antivir. Res. 53 (A66), 104.

Jackson, R.J., Ramsay, A.J., Christensen, C.D., Beaton, S., Hall, D.F., Ramshaw, I.A., 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. J. Virol. 75, 1205–1210.

Kern, E.R.C., Hartline, C., Harden, E., Keith, K., Rodriguez, N., Beadle, J.R., Hostetler, K.Y., 2002. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob. Agents Chemother. 46, 991–995.

Lalezari, J.P., Stagg, R.J., Kuppermann, B.D., Holland, G.N., Kramer, F., Ives, D.V., Youle, M., Robinson, M.R., Drew, W.L., Jaffe, H.S., 1997. Intravenous cidofovir for peripheral cytomegalovirus retinitis in patients with AIDS. A randomized, controlled trial. Ann. Intern. Med. 126, 257–263.

Martinez, M.J., Bray, M.P., Huggins, J.W., 2000. A mouse model of aerosol-transmitted orthopoxviral disease. Arch. Pathol. Lab. Med. 124, 362–377.

Smee, D.F., Bailey, K.W., Sidwell, R.W., 2001. Treatment of lethal vaccinia virus respiratory infections in mice with cidofovir. Antivir. Chem. Chemother. 12, 71–76.

Snoek, R., Sakuma, T., De Clercq, E., Rosenberg, I., Holy, A., 1988. (S)–1-(3-Hydroxy-2-phosphonomethoxypropyl)cytosine, a potent and selective inhibitor of human cytomegalovirus replication. Antimicrob. Agents Chemother. 32, 1839–1844.

The Studies of Ocular Complications of AIDS Research Group in collaboration with The AIDS Clinical Trials Group, Cidofovir (HPMPC) for the treatment of cytomegalovirus retinitis in patients with AIDS: the HPMPC peripheral cytomegalovirus retinitis trial. Ann. Int. Med. 126, 264–274.

Tojo, A., Sekine, T., Nakajima, N., Hosoyamada, M., Kanai, Y., Kimura, K., Endou, H., 1999. Immunohistochemical localization of multispecific renal organic anion transporter 1 in rat kidney. J. Am. Soc. Nephrol. 10, 464–471.

Wachsman, M., Petty, B.G., Cundy, K.C., Jaffe, H.S., Fisher, P.E., Pastelak, A., Lietman, P.S., 1996. Pharmacokinetics, safety and bioavailability of HPMPC (cidofovir) in human immunodeficiency virus-infected subjects. Antivir. Res. 29, 153–161.

Xiong, X., Smith, J.L., Kim, C., Huand, E., Chen, M.S., 1996. Kinetic analysis of the interaction of cidofovir diphosphate with human cytomegalovirus DNA polymerase. Biochem. Pharmacol. 51, 1563–1567.

Notes to Authors

For a full and complete Instructions To Authors, please refer to *Antiviral Research*, Vol. 59, No. 1, pp. v–vii.
The instructions can also be found on the World Wide Web: access under http://www.elsevier.com

Electronic manuscripts: Electronic manuscripts have the advantage that there is no need for the rekeying of text, thereby avoiding the possibility of introducing errors and resulting in reliable and fast delivery of proofs.

For the initial submission of manuscripts for consideration, hardcopies are sufficient. For the processing of *accepted papers* electronic versions are preferred. After *final acceptance* your disk plus three, final and exactly matching printed versions should be submitted together. Double density (DD) or high density (HD) diskettes ($3\frac{1}{2}$) are acceptable. It is important that the file saved is in the native format of the wordprocessor program used. Label the disk with the name of the computer and wordprocessing package used, your name, and the name of the file on the disk. Further information may be obtained from the Publisher.

Authors in Japan please note: Upon request, Elsevier Japan will provide authors with a list of people who can check and improve the English of their paper (*before submission*). Please contact our Tokyo office: Elsevier Japan, 4F Higashi-Azabu, 1-Chome Bldg, 1-9-15 Higashi-Azabu, Minato-ku, Tokyo 106-0044, Japan, Tel.: (+81) (3) 5561 5037; Fax: (+81) (3) 5561 5047.

Author enquiries
Authors can keep a track on the progress of their accepted article, and set up e-mail alerts informing them of changes to their manuscript's status, by using the "Track a Paper" feature of Elsevier's Author Gateway (http://www.authors.elsevier.com)

Full details for the electronic submission of artwork can be obtained from http:/www.elsevier.com/locate/authorartwork/

Contact details for questions arising after acceptance of an article, especially those relating to proofs, are provided after registration of an article for publication.

***Antiviral Research* has no page charges**

USA mailing notice: Antiviral Research (ISSN 0166-3542) is published monthly by Elsevier B.V. (P.O. Box 211, 1000 AE Amsterdam, The Netherlands). Annual subscription price in the USA US$ 1912 (valid in North, Central and South America), including air speed delivery. Periodical postage rate paid at Jamaica, NY 11431.
USA POSTMASTER: Send address changes to Antiviral Research, Publications Expediting Inc., 200 Meacham Ave, Elmont, NY 11003.
AIRFREIGHT AND MAILING in the USA by Publications Expediting Inc., 200 Meacham Avenue, Elmont, NY 11003.

Printed in the United Kingdom

⊘ The paper in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper).

In Press, Int. J. Antimicrob. Chemotherap.

Effects of highly active antiviral substances on lethal vaccinia virus (IHD strain) respiratory infections in mice

Donald F. Smee[a,*], Min-Hui Wong[a], Kevin W. Bailey[a], James R. Beadle[b], Karl Y. Hostetler[b], Robert W. Sidwell[a]

[a] Institute for Antiviral Research, Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, UT 84322, USA

[b] Department of Medicine, San Diego VA Healthcare System and the University of California, San Diego, La Jolla, CA 92093-0676, USA

* Corresponding author.

Tel: +1 435 797 2897; Fax: +1 435 797 3959;

E-mail address: dsmee@cc.usu.edu (D.F. Smee)

Abstract

Intranasal infection of BALB/c mice with the IHD strain of vaccinia virus was found to cause pneumonia, profound weight loss, and death. Cidofovir, hexadecyloxypropyl-cidofovir (HDP-CDV), the diacetate ester prodrug of 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (HOE961) and ribavirin were used to treat the infections starting 24 h after virus exposure. Single intraperitoneal (i.p.) cidofovir treatments of 100 and 30 mg/kg led to 90-100% survival compared to no survivors in the placebo group, whereas a 10 mg/kg dose was ineffective. The 100 mg/kg treatment reduced lung and snout virus titers on day 3 of the infection by 20- and 8-fold, respectively. Mean arterial oxygen saturation levels in these two cidofovir treatment groups were significantly higher than placebo on days 4 through 6 of the infection, indicating an improvement in lung function. Effects of cidofovir on viral pathogenesis were studied on days 1, 3, and 5 of the infection, and demonstrated statistically significant reductions in lung consolidation scores, lung weights, lung virus titers, and snout virus titers on days 3 and 5. Cidofovir treatment also reduced virus titers in other tissues and body fluid, including blood, brain, heart, liver, salivary gland, and spleen. HDP-CDV was given orally at 100, 50 and 25 mg/kg one time only, resulting in 80-100% survival. Lower daily oral doses of 10 and 5 mg/kg/day given for 5 days protected only 30% of animals from death. Oral doses (100, 50, and 25 mg/kg/day) of HOE961 for 5 days protected all animals, whereas equivalent oral doses of ribavirin were completely ineffective. The rapidity of recovery from weight loss during the infection was a function of dose of compound administered. These data indicate the utility

of parenteral cidofovir, oral HDP-CDV and oral HOE961 in treating severe respiratory infections caused by this virus.

Keywords: vaccinia virus, pathogenesis, cidofovir, HDP-CDV, HOE961, ribavirin, antiviral

1. Introduction

Efforts are intensifying to discover and develop compounds potentially active against the bioterrorism viruses variola (smallpox) and monkeypox [1-4]. The treatment of progressive vaccinia as a complication of vaccination in immunodeficient individuals [5] or of other poxvirus infections such as molluscum contagiosum [6-9] or orf virus [10] may also be advanced by this research.

Animal models of lethal respiratory virus infections in mice have been used to evaluate known-active antiviral agents with potential utility against pox viruses [11]. Cowpox virus was shown by Bray et al. [12] to cause fatal infections in mice when given by intranasal or aerosol challenges. Martinez et al. [13] reported the pathology of the virus in these animals. This model has been used to demonstrate the antiviral activities of cidofovir [12, 14], ribavirin [15], 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (S2241) [16], and the diacetate ester prodrug (HOE961) of S2242 [16]. More recently, alkoxyalkyl esters of cidofovir such as hexadecyloxypropyl-cidofovir (HDP-CDV), have been reported to be effective orally against cowpox virus infections in mice [17].

Nelson [18] first reported that vaccinia virus would cause lethal infections in mice when administered intranasally. Turner [19] showed that the WR strain of vaccinia virus was the

most lethal by this inoculation route. He also reported that the IHD strain caused some deaths in mice, whereas the Copenhagen, Lister, Levaditi, and Tashkent strains of vaccinia were not lethal by intranasal instillation. Early chemotherapy studies using the intranasal infection model of vaccinia virus were conducted with either the WR [20, 21] or IHD [22] strains. These experiments identified the potential utility of thiosemicarbazones and phenoxythiouracils as anti-poxvirus agents. Smee et al. [23, 24] used the vaccinia virus (WR strain) intranasal model to demonstrate the efficacy of cidofovir in the treatment of the infection.

Based upon Turner's [19] work, it was initially thought that the IHD strain was much less virulent than the WR strain and may not be adequate for a lethal mouse infection model. However, Thompson et al. [22] successfully employed the IHD strain in a chemotherapy experiment. Our approach was to produce a very high titer pool of the IHD virus strain and determine its lethal dose in mice. From those evaluations it was determined that the IHD strain was much more virulent than anticipated, in fact it was similar in virulence to the WR strain.

The activity of cidofovir was well characterized against infections with the WR strain of virus [23, 24] using intraperitoneal treatment (the compound is ineffective when given orally). It was deemed appropriate to study the drug against intranasal infections in mice caused by the IHD virus strain. HDP-CDV is the hexadecyloxypropyl ester of cidofovir and it exhibits a 58 to 74-fold increase in antiviral activity against vaccinia virus, Copenhagen or Brighton strains, *in vitro* [25]. The increased antiviral activity of HDP-CDV has been shown to be due to greatly increased cellular uptake and conversion to cidofovir diphosphate, the

active antiviral metabolite [A]. Esterification of CDV to HDP-CDV also promotes excellent oral bioavailability, 88%, in mice versus <5% for cidofovir [B][C]. We also wanted to assess the efficacy of two other compounds with potential oral activity in the model, HOE961, and ribavirin. Up to the present time, only parenteral cidofovir has been studied in both cowpox and vaccinia models, with no published studies of other compounds against vaccinia virus.

2. Materials and Methods

2.1. Antiviral compounds

Mick Hitchcock of Gilead Sciences (Foster City, CA, USA) kindly provided cidofovir. Ribavirin was from ICN Pharmaceuticals (Costa Mesa, CA, USA). HDP-CDV was synthesized from cyclic cidofovir by coupling to hexadecyl bromide as reported previously [25]. Cyclic cidofovir was generously provided by Gilead Sciences, Foster City, CA. HOE961 was kindly provided by Juergen Puenter of Aventis Pharma (Frankfurt, Germany). Cidofovir was dissolved in sterile saline for intraperitoneal (i.p.) injection into mice. Sterile water was used to dissolve the other compounds for oral gavage treatments. Sterile saline or water served as the respective placebo control.

2.2. Virus and cells

Vaccinia virus (IHD strain) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The virus was propagated in African green monkey kidney (MA-104) cells (BioWhittaker, Walkersville, MD, USA). Plaque assays of the virus were

done in African green monkey kidney (Vero) cells (from ATCC). The MA-104 cells were cultured in Eagle's medium (MEM) containing 9% fetal bovine serum (FBS) whereas the Vero cells were grown in Medium 199 with 5% FBS. MEM with 2% FBS and gentamicin (50 μg/ml) was used for viral propagation and plaque assays.

2.3. Mouse infection studies

Female BALB/c mice (13-15 g) were purchased from either B & K Universal (Fremont, CA, USA) or Simonsen Laboratories (Gilroy, CA, USA) for the studies. An infectious vaccinia virus challenge of 5×10^5 to 1×10^6 plaque forming units (about 10 50% lethal doses) per mouse was used for the experiments. This was based upon previously conducted lethality titrations with the virus in mice. Virus was administered intranasally in a 50 μl volume following anesthesia with ketamine (100 mg/kg given by i.p. injection). Infection under anesthesia (as opposed to application to non-narcotized animals) allows the infectious fluid to penetrate deeper into the lungs, and fewer virus particles are required to achieve a 50% lethal dose. Treatment regimens were based upon published results for cidofovir [12, 23, 24], ribavirin [15], and HOE961 [16]. The single treatment regimen using HDP-CDV was modeled after cidofovir. Treatments with HDP-CDV for 5 days was done using the maximum tolerated dose of 10 mg/kg/day. Animals were individually weighed every 2-3 days and deaths recorded for 21 days. There were 10 mice per group held for death and 5 mice per group per day (when used) for virus titer determinations. Cidofovir, HOE961, and ribavirin have previously been reported to be non-toxic at the doses and regimens used. The toxicity of HDP-CDV will be addressed in this report. Uninfected

toxicity control mice (5 per group) were used for assessing toxicity, with animals weighed during and after the treatment period to assess drug tolerability.

Lung infection parameters were determined in a manner similar to those reported for influenza virus [26] and vaccinia virus (WR strain) [23, 24] in groups of infected mice. On days 1, 3 and 5 of the infection, lungs from sacrificed mice were collected, given a severity score based upon lung discoloration ranging from 0 (normal) to 4 (100% of lung area exhibiting a plum discoloration), weighed, and frozen for later virus titration. Other tissues and whole blood (placed in 0.5 ml of cell culture medium and vortexed) were taken for virus titer determinations. The samples were frozen at –80°C prior to homogenization and titration. Virus titers from these samples were later determined by plaque assay in Vero cells as described previously [23, 24].

Arterial oxygen saturation (SaO_2) levels (a measure of lung function) were determined on days 2-10 of the infection using a pulse oximetry method [27]. Animals dead on the particular day of measurement were assigned an SaO_2 value of 75 for that day, since this value was the lowest that we have observed in mice near death.

2.4. Statistical methods

Statistical comparisons were made of the drug-treated groups to the placebo control by two-tailed analyses. The Fisher exact test was used to interpret differences in numbers of survivors. Mean day of death, mean body weight area under the curve (for days 5 through 21 of the infection), mean tissue or blood virus titers, mean lung scores, mean lung weight comparisons, and mean SaO_2 level differences were statistically analyzed by the Mann-

Whitney U-test. Calculations were made using the InStat computer program (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effect of cidofovir treatment on the infection

Mice were infected intranasally with the IHD strain of vaccinia virus and treated i.p. one day later with cidofovir (100, 30, and 10 mg/kg) or placebo. Treatment with 100 and 30 mg/kg resulted in 100 and 90% survival, respectively, whereas the 10 mg/kg dose was not protective (Table 1). A moderate 1.3 day increase in the mean day of death was observed in the 10 mg/kg group. Lung and snout virus titers were assessed on day 3 of this experiment. Statistically significant decreases in virus titers were seen in the 100 and 30 mg/kg groups. Lung and snout virus titers decreased 20- and 8-fold in the 100 mg/kg group compared to placebo but only about 3-fold in the 30 mg/kg group. Very small decreases in virus titers were seen in the 10 mg/kg group. Mean body weights that accompany the study in Table 1 are presented in Figure 1A. Weight losses were evident in all treated groups through day 7. After that time, animals in the 100 and 30 mg/kg groups recovered from the infection and their weights increased steadily. The rates of weight gain from day 10 onward were similar among treated and uninfected groups, since the lines were parallel although the initial starting weights on day 10 differed. The area under the curve from days 10 through 21 for the 100 mg/kg group differed significantly from that of the 30 mg/kg group ($P<0.03$), indicating the mice fared better during the infection and recovery phase.

Arterial oxygen saturation (SaO_2) levels were assessed from the onset of the infection as a measure of lung function (Figure 1B). Decreased SaO_2 levels were noted in the placebo group on days 4 through 6. The 10 mg/kg treatment group showed parallel declines to the placebo group starting approximately two days later. There was a large mouse-to-mouse variation in SaO_2 levels at each time point. A significant difference in SaO_2 values was evident between placebo and the 30 mg/kg group on day 4, and between the placebo compared to the 100, 30, and 10 mg/kg groups on days 5 and 6. By day 6 the placebo-treated mice were all dead, and no further statistical comparison were made after that time.

The effects of cidofovir treatment on viral pathogenesis were studied on days 1, 3, and 5 of the infection in a separate experiment. Lung consolidation scores and lung weights were dramatically elevated in the placebo group on day 5 (Figures 2A and 2B). All doses of the compound significantly reduced these infection parameters, with 10 mg/kg showing the weakest effect. Lung virus titers were approaching 10^7 plaque forming units per gram on day 1 before the cidofovir treatment was given (Figure 2C); about 100-fold less virus was present in the snout (Figure 2D). Cidofovir suppressed lung and snout virus titers in a dose-dependent manner, with the 10 mg/kg dose being only marginally active.

A third experiment was conducted with cidofovir to determine the effects of treatment on virus titers in various tissues and blood (Table 2). On day 5 of the infection (a day before the onset of death of placebo-treated mice), high virus titers were found in lung, snout, and spleen. Lower, but appreciable amounts of virus were recovered from blood, brain, heart, kidney, liver, and salivary gland. The stomach and intestines had little or no virus present. Cidofovir treatment effectively reduced virus titers in all of these tissues and blood. Lung

and snout (the primary sites of infection) virus titers were reduced to a lesser extent than were all of the secondary sites of infection, except brain.

3.2. Effect of oral HDP-CDV treatment on the infection

HDP-CDV was administered orally to mice one time only at 24 h after IHD virus exposure (Table 3). Doses of 100, 50, or 25 mg/kg/day protected 80-100% of the mice from mortality. The two mice that died in the 25 mg/kg group lived 10 days longer than placebo control animals. Cidofovir at 100 mg/kg given by i.p. administration was run as a positive control and protected 90% of the animals from death. Weight loss during the acute phase of the infection (days 1-9) and weight gain during the recovery phase (days 10-21) are presented in Figure 3A. The pattern of weight loss and recovery for the 100 and 50 mg/kg doses were similar to the weight pattern resulting from cidofovir treatment, indicating equal efficacy. Weight loss in the 25 mg/kg HDP-CDV treated mice was more severe than in the other groups. After the acute phase of the infection had passed, the rate of weight gain was rapid.

Doses of 10, 5, and 2.5 mg/kg/day of HDP-CDV were given for 5 consecutive days The 30% survival rate due to 10 and 5 mg/kg/day treatments were not statistically significant. The mean days of death were significantly increased with the three dosages of compound relative to the placebo control, although only minimally by treatment with the 2.5 mg/kg/day dose. Weight loss and gain patterns during the infection are presented in Figure 3B. The degree of weight loss during the acute infection was as severe or more severe as seen in the single dose 25 mg/kg group (Figure 3A). Weight gain following the

acute infection proceeded more slowly in the 5 mg/kg/day group than in the 10 mg/kg/day group indicating better efficacy at the higher dose.

Toxicity evaluations in uninfected mice revealed that HDP-CDV was more toxic than cidofovir. In uninfected animals, single oral treatments with HDP-CDV at 25, 50, and 100 mg/kg resulted in weight changes of –0.4, -1.3, and –2.0 g over a 2-day period, respectively, compared to a weight gain of 0.8 g in the placebo group and 0.8 g in the 100 mg/kg cidofovir group. Daily oral doses of HDP-CDV for 5 days at 2.5, 5, and 10 mg/kg/day resulted in weight changes over a 6-day period of +1.2, +0.7, and –0.7 g, compared to +0.8 g in the placebo group. Thus, single doses (25, 50, and 100 mg/kg) and a daily dose (10 mg/kg/day) of HDP-CDV were not well tolerated by the mice, based upon weight loss. Mice quickly regained weight after cessation of treatment, however.

3.3. Effect of HOE961 treatment on the infection

Oral treatments twice a day for 5 days with HOE961 at 100, 50, or 25 mg/kg/day resulted in 100% survival from the IHD virus infection in mice (Table 3). Animal weights during the infection are shown in Figure 3C. The 100 mg/kg/day dose of HOE961 was equal in efficacy to the 100 mg/kg i.p. dose of cidofovir. Each succeeding lower dose of HOE961 was less protective to the animals, based upon extent of weight loss during the acute infection and resulting time to regain the weight.

3.4. Effect of ribavirin treatment on the infection

Twice-daily oral ribavirin treatments of 100, 50, and 25 mg/kg/day were not protective against the lethal infection (Table 3). The highest dose caused a slight delay in the time to death. Weight losses during the infection were severe, as depicted in Figure 3D.

Ribavirin was also evaluated by i.p. route using 100 mg/kg/day for 5 days starting 24 h after virus challenge. Several half-$\log_{10}$ infecting virus doses were used ranging from 10^3 to 10^6 PFU/mouse. Doses below $10^{4.5}$ PFU/mouse were not lethal to placebo-treated animals. Under none of the infection conditions where mice died was ribavirin able to protect animals from death. Lung virus titers from mice sacrificed on day 5 of the infection were reduced no more than four-fold (usually less) by ribavirin treatment, even when sub-lethal infecting virus doses were given (data not shown).

4. Discussion

The vaccinia (IHD strain) respiratory infection model shares many features in common with the vaccinia (WR strain) model reported previously [23, 24]. Using comparable virus challenge doses, lung and snout virus titers rose to high levels over a similar time course, as did mean lung weights and lung consolidation scores. In the infection with the WR strain, virus was found in the intestine, and was at a higher level in the blood and stomach than with the IHD strain infection. Virus titers in the brain, heart, kidney, liver, lung, snout, salivary gland, and spleen were very similar between WR and IHD strain infections. Both infections induced rapid body weight loss, the animals died quickly after infection, and measurable losses of lung function occurred (as determined by pulse oximetry). As was indicated for the WR virus infection, even though there was virus detected in the brain

following infection with the IHD strain, the animals did not exhibit symptoms (tremors, limb paralysis, leaning to one side) that would suggest encephalitis as being the cause of death.

The responses of mice to cidofovir treatment during infections caused by the WR and IHD virus strains were similar, comparing these to previous results [23, 24]. In both infections the 100 and 30 mg/kg doses administered one time only 24 h after virus exposure resulted in 80-100% protection from mortality. Body weights in treated groups dropped precipitously during both infections, and the survivors began to regain weight after a week of illness. The extent of virus titer suppression due to drug treatment in lungs and snout were about the same for both infections, as were effects on lung consolidation scores and lung weights. Cidofovir treatment reduced virus titers in the lungs and snout (primary sites of infection) to a lesser extent than it reduced virus in other tissues and blood (secondary sites of infection) in the WR and IHD infection models. At the primary sites each virus replicated for one day prior to exposure to the drug. The secondary sites had negligible virus in them on day 1 when the drug was administered, hence cidofovir was presumably present before the exposure of these areas to the virus. This could explain why virus inhibition was greater in the secondary sites. The exception to this was the brain, where the effect of cidofovir on virus titer reduction was less dramatic. Penetration of the drug into the brain may have been less efficient than it was in other parts of the body.

In both infections, cidofovir treatment helped prevent the decline of arterial oxygen saturation that occurred during the latter part of the infection in the placebo groups.

The measurement of arterial oxygen saturation was found to be a useful parameter only under conditions where high virus challenge doses were given. This applies to either the WR or IHD strain infection model. Mice receiving lower virus challenges did not exhibit large increases in lung weight and lung consolidation scores, and their arterial oxygen saturation remained near the normal range even though the animals died. This is because in these models (and in the cowpox respiratory infection model), the cause of death does not need to be pneumonitis. We have previously indicated that cowpox virus can be lethal when administered by i.p. challenge, as are high doses of WR and IHD strains of vaccinia virus [11]. There is toxemia associated with these poxvirus infections that leads to death in the absence of pneumonitis. Because the vaccinia virus-infected mice died so rapidly, there were few days in which to compare pulse oximetry measurements between the placebo and drug-treated groups. In the present study, days 4 through 6 after virus exposure were useful for statistically comparing SaO_2 measurements among treatment groups.

HDP-CDV proved to be effective orally in treating the IHD virus infection when given as single treatments. Lower oral doses given over five days was found to be weakly effective, however. Toxicity prevented using the compound at higher daily doses, and the single high doses caused weight loss over a two-day period. The molecular weight of HDP-CDV is almost exactly twice that of cidofovir, which means that the equivalent doses of cidofovir delivered by treatment with HDP-CDV would be one-half of the mg/kg values reported on the tables and in the text. HDP-CDV is more active and selective against orthopoxviruses than cidofovir in cell culture, but exhibits greater cytotoxicity as well because of the greater extent of cell uptake and coversion to CDV-diphosphate [25][A].

Formal toxicity testing of HDP-CDV in animals will provide more definitive information about mechanisms and significance of drug side effects.

We have conducted several other studies using HDP-CDV to treat infections caused by the WR strain of vaccinia virus (unpublished). This has included experiments similar to those reported in Table 3. We have also used a 20 mg/kg/day loading dose for 2 days followed by lower doses for 3 more days. In each case, HDP-CDV was unable to prevent death of the animals. Thus, there is a difference in the nature of the WR versus the IHD virus infections, with the WR infection being more difficult to treat. It may possibly be related to the WR strain being more neurovirulent than the IHD strain.

The antiviral activity of HOE961 in the IHD virus infection model was not surprising, based upon its efficacy against cowpox virus infections in mice [16]. The advantage that this compound and HDP-CDV may have is that they are orally active, whereas cidofovir requires i.p. administration (or intravenous treatment of humans). HDP-CDV may not be the most active of its type, however. A recent report indicates that octadecyloxyethyl-cidofovir (ODE-cidofovir) may be more effective than HDP-CDV in treating orthopoxvirus infections in animals [17]. In addition, several other ether lipid analogs are being evaluated as potential oral therapies for orthopoxvirus infections in animal models of disease (K.Y.Hostetler, unpublished, 2003).

The efficacy of ribavirin in the cowpox virus infection model [15] did not translate into similar efficacy in the vaccinia virus model, even against sub-lethal infections.
Besides being beneficial against cowpox virus infections, the compound was also previously shown to suppress vaccinia tail lesions in mice when given by i.p. route [28] and to be

effective in treating vaccinia keratitis in rabbits by topical application [29]. Ribavirin combined with vaccinia immunoglobulin helped to arrest progressive vaccinia in an immunocompromised individual [5] in an uncontrolled trial. The potential of ribavirin as a single agent in treating orthopoxvirus infections seems doubtful based upon the current findings.

These studies support the utility of the vaccinia virus (IHD strain) infection model for antiviral studies. It is a slightly less severe infection than that caused by the WR strain, based upon differential efficacy patterns of HDP-CDV against these viral strains. From the investigations with the IHD strain, it is concluded that parenterally-administered cidofovir or orally administered HDP-CDV and HOE961 are effective in treatment of the infection. Other orally active derivatives of cidofovir, such as ODE-cidofovir, should be studied in this infection model.

Acknowledgements

This work was supported by contracts N01-AI-65291 and NO1-AI-15435 from the Virology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health and by NIH grants EY11832, AI12964 and by Department of the Army Grant 17-01-2-0071. The U.S. Army Medical Research Acquisition Activity, Ft. Detrick, MD, is the awarding acquisition office. The contents of this article do not necessarily reflect the position or policy of the government and no official endorsement should be inferred.

References

[A] Aldern, K.A., Ciesla, S.L., Winegarden, K.L. and Hostetler, K.Y., The increased antiviral activity of 1-O-hexadecyloxypropyl-cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism, Molecular Pharmacology, 63:678-681, 2003.

[B] Ciesla, S.L., Trahan, J., Winegarden, K.L., Aldern, K.A., Painter, G.R. and Hostetler, K.Y., Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney, Antiviral Research, in press, 2003.

[C] Wachsman M, B.G.Petty K.C. Cundy, H.S. Jaffe, P.E. Fisher, A. Pastelak and P.S. Lietman. 1996. Pharmacokinetics, safety and bioavailability of HPMPC (cidofovir) in human immunodeficiency virus-infected subjects, Antiviral Res., 29:153-61.

[1] Breman JG, Henderson DA. Poxvirus dilemmas - monkeypox, smallpox and biological terrorism. N Engl J Med 1998;339:556-9.

[2] Hooper C. Poxvirus dilemmas. N Engl J Med 1998;339:2027-8.

[3] Orent W. Escape from Moscow. Sciences (New York) 1998;38:26-31.

[4] Peters CJ. Many viruses are potential agents of bioterrorism. ASM News 2002;68: 168-73.

[5] Kesson AM, Ferguson JK, Rawlinson WD, Cunningham AL. Progressive vaccinia treated with ribavirin and vaccinia immune globulin. Clin Infect Dis 1997;25:911-4.

[6] Meadows KP, Tyring SK, Pavia AT, Rallis TM. Resolution of recalcitrant molluscum contagiosum virus lesions in human immunodeficiency virus-infected patients treated with cidofovir. Arch Dermatol 1997;133:987-90.

[7] Davies EG, Thrasher A, Lacey K, Harper J. Topical cidofovir for severe molluscum contagiosum. Lancet 1999;353:2042.

[8] Ibarra V, Blanco JR, Oteo JA, Rosel L. Efficacy of cidofovir in the treatment of recalcitrant molluscum contagiosum in an AIDS patient. Acta Derm-Venereol 2000;80:315-6.

[9] Toro JR, Wood LV, Patel NK, Turner ML. Topical cidofovir: a novel treatment for recalcitrant molluscum contagiosum in children infected with human immunodeficiency virus 1. Arch Dermatol 2000;136:983-5.

[10] Geerinck K, Lukito G, Snoeck R, De Vos R, De Clercq E, Vanrenterghem Y, Degreef H, Maes B. A case of human orf in an immunocompromised patient treated successfully with cidofovir cream. J Med Virol 2001;64:543-9.

[11] Smee DF, Sidwell RW. A review of compounds exhibiting anti-orthopoxvirus activity in animal models. Antiviral Res 2003;57:41-52.

[12] Bray M, Martinez M, Smee DF, Kefauver D, Thompson E, Huggins JW. Cidofovir (HPMPC) protects mice against lethal aerosol or intranasal cowpox virus challenge. J Infect Dis 2000;181:10-9.

[13] Martinez MJ, Bray MP, Huggins JW. A mouse model of aerosol-transmitted orthopoxviral disease: morphology of experimental aerosol-transmitted orthopoxviral disease in a cowpox virus-BALB/c mouse system. Arch Pathol LabMed 2000;124:362-7.

[14] Smee DF, Bailey KW, Wong M-H, Sidwell RW. Intranasal treatment of cowpox virus respiratory infections in mice with cidofovir. Antiviral Res 2000;47:171-7.

[15] Smee DF, Bailey KW, Sidwell RW. Treatment of cowpox virus respiratory infections in mice with ribavirin as a single agent or followed sequentially by cidofovir. Antiviral Chem Chemother 2000;11:303-9.

[16] Smee DF, Bailey KW, Sidwell RW. Treatment of lethal cowpox virus respiratory infections in mice with 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine and its orally active diacetate ester prodrug. Antiviral Res 2002;54:113-20.

[17] Quenelle DC, Collins DJ, Herod BP, Beadle JR, Wan WB, Hostetler KY, Kern ER. Effect of oral treatment with alkoxyalkyl esters of cidofovir on cowpox or vaccinia virus infections in mice. Antiviral Research 2003;57:A79.

[18] Nelson JB. The behavior of pox viruses in the respiratory tract. I. The response of mice to the nasal instillation of vaccinia virus. J Exp Med 1938;68:401-12.

[19] Turner GS. Respiratory infection of mice with vaccinia virus. J Gen Virol 1967;1: 399-402.

[20] Hamre D, Bernstein J, Donovick R. Activity of *p*-aminobenzaldehyde, 3-thiosemicarbazone on vaccinia virus in the chick embryo and in the mouse. Proc Soc Exp Biol Med 1950;73:275-8.

[21] Hamre D, Brownlee KA, Donovick R. Studies on the chemothrapy of vaccinia virus. II. The activity of some thiosemicarbazones. J Immunol 1951;67:305-12.

[22] Thompson RL, Price M, Minton SA Jr, Falco EA, Hitchings GH. Protection of mice against the vaccinia virus by the administration of phenoxythiouracils. J Immunol 1951;67:483-91.

[23] Smee DF, Bailey KW, Sidwell RW. Treatment of lethal vaccinia virus respiratory infections in mice with cidofovir. Antiviral Chem Chemother 2001;12:71-6.

[24] Smee DF, Bailey KW, Wong M-H, Sidwell RW. Effects of cidofovir on the pathogenesis of a lethal vaccinia virus respiratory infection in mice. Antiviral Res 2001;52:55-62.

[25] Kern ER, Hartline C, Harden E, Keith K, Rodriguez N, Beadle JR, Hostetler KY. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob Agents Chemother 2002;46:991-5.

[26] Sidwell RW, Huffman JH, Barnard DL, Bailey KW, Wong M-H, Morrison A, Syndergaard T, Kim CU. Inhibition of influenza virus infection s in mice by GS4104, an orally effective influenza virus neuraminidase inhibitor. Antiviral Res 1998;37:107-20.

[27] Sidwell RW, Huffman JH, Gilbert,J, Moscon B, Pedersen G, Burger R, Warren RP. Utilization of pulse oximetry for the study of the inhibitory effects of antiviral agents on influenza virus in mice. Antimicrob Agents Chemother 1992;36:473-6.

[28] De Clercq E, Luczak M, Shugar D, Torrence PF, Waters JA, Witkop B. Effect of cytosine arabinoside, iododeoxyuridine, ethyldeoxyuridine, thiocyanatodeoxyuridine, and ribavirin on tail lesion formation in mice infected with vaccinia virus. Proc Soc Exp Biol Med 1976;151:487-90.

[29] Sidwell RW, Allen LB, Khare GP, Huffman JH, Witkowski JT, Simon LN, Robins RK. Effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on

herpes and vaccinia keratitis and encephalitis in laboratory animals. Antimicrob Agents Chemother 1973;3:242-6.

Figure 1. Effects of i.p. treatment with cidofovir on mean body weights (A) and on arterial oxygen saturation (SaO_2) levels (B) during a vaccinia virus (IHD strain) respiratory infection in mice. Treatments were given one time only at 24 h after virus exposure. Error bars indicate standard deviations (10 mice/group). Symbols: placebo (E); cidofovir at 10 (H), 30 (B), or 100 (J) mg/kg; uninfected (G). *P<0.05, ** P<0.01, *** P<0.001.

Figure 2. Effects of i.p. treatment with cidofovir on mean lung scores (A), lung weights (B), lung virus titers (C), and snout virus titers (D) during a vaccinia virus (IHD strain) respiratory infection in mice. Treatments were given one time only at 24 h after virus exposure. Error bars indicate standard deviations (5 mice/group). Virus titers are expressed as PFU/g. Symbols: placebo (E); cidofovir at 10 (H), 30 (B), or 100 (J) mg/kg; uninfected (G). *P<0.05, ** P<0.01, *** P<0.001.

Figure 3. Effects of treatment with compounds on mean body weights during a vaccinia virus (IHD strain) respiratory infection in mice. All treatments started 24 h after virus exposure. Symbols: Panel A = one oral treatment with HDP-CDV at 25 (F), 50 (H), or 100 (C) mg/kg; Panel B = oral treatments with HDP-CDV twice a day for 5 days at 2.5 (F), 5 (H), or 10 (C) mg/kg/day; Panels C and D = oral treatments with HOE961 and ribavirin, respectively, twice a day for 5 days at 25 (F), 50 (H), or 100 (C) mg/kg/day; oral placebo (E) and i.p. cidofovir at 100 mg/kg (J) given as single injections apply to all figures.

#5

Antimicrobial Agents & Chemotherapy, in press, 2003

Oral Treatment of Cowpox and Vaccinia Infections in Mice with Ether Lipid Esters of Cidofovir

Running Title: Ether lipid esters of cidofovir treatments of poxvirus infections

D.C. Quenelle[1], D.J. Collins[1], K.Y. Hostetler[2], J.R. Beadle[2], W.B. Wan[2] and Earl R. Kern[1]

[1]The University of Alabama School of Medicine, Birmingham, Ala., USA

[2]The San Diego VA Healthcare System and the University of California, San Diego, La Jolla, California, USA

Corresponding Author:
Earl R. Kern, Ph.D.
The University of Alabama at Birmingham
Department of Pediatrics
128 Children's Harbor Building
1600 6th Avenue South
Birmingham, AL 35233
Ph. (205) 934-1990
Fax (205) 975-1992
E-mail: Kern@uab.edu

Presented at the 16th ICAR, Savannah, GA April 27-30, 2003

Abstract

Four newly synthesized alkoxyalkyl esters of cidofovir (CDV): hexadecyloxypropyl-CDV (HDP-CDV), octadecyloxyethyl-CDV (ODE-CDV), oleyloxyproplyl-CDV (OLP-CDV) and oleyloxyethyl-CDV (OLE-CDV), were found to have enhanced activities against vaccinia and cowpox viruses *in vitro*. The compounds were administered orally and evaluated for efficacy against lethal cowpox virus (CV) or vaccinia virus (VV) infections in mice. HDP-CDV, ODE-CDV or OLE-CDV was effective at preventing mortality to CV when treatments were initiated 24 h post viral inoculation, but only HDP-CDV and ODE-CDV maintained efficacy when treatments were initiated as late as 72 h. Oral pretreatment with HDP-CDV or ODE-CDV was also effective when given 5, 3, or 1 day prior to virus inoculation with CV, even when administered as a single dose. Both HDP-CDV or ODE-CDV were also effective against VV infections when administered orally 24 or 48 h after infection. In animals treated with HDP-CDV or ODE-CDV, virus titers in liver, spleen and kidney were reduced 3-7 $\log_{10}$ for both CV and VV. In contrast, virus replication in lung was not significantly reduced. These data indicate that HDP-CDV or ODE-CDV given orally is as effective as parenteral CDV for the treatment of experimental CV and VV infections and suggests that the compounds may be useful for treatment of orthopoxvirus infection in humans.

Introduction

Orthopoxvirus diseases continue to pose challenges to researchers preparing for a bioterrorist release of biological weapons of mass destruction. Rapid diagnostics for smallpox and development of effective antiviral chemotherapies are two essential components for national preparedness (16,17). While cidofovir is well documented for its *in vitro* and *in vivo* activity against orthopoxviruses (3, 4, 8, 9, 15, 18, 20-24), its usefulness is limited by the requirement for intravenous administration and the associated nephrotoxicity (14). Orally active compounds with prolonged therapeutic blood levels and reduced toxicity would prove to be optimal for mass distribution in response to an actual release of smallpox.

Results from earlier studies using acyclovir and ganciclovir showed that alkoxyalkyl esters of the monophosphates of ganciclovir or acyclovir had improved oral bioavailability compared to the unmodified parent compounds and were effective against cytomegalovirus and herpes simplex virus infections (13). To improve oral bioavailability of CDV, a novel series of analogs of CDV were synthesized by esterification with long-chain alkoxyalkanols. Hexadecyloxypropyl-CDV (HDP-CDV), and octadecyloxyethyl-CDV (ODE-CDV) were

synthesized and evaluated *in vitro* for efficacy against cowpox and vaccinia virus infections. As previously reported, HDP-CDV had EC_{50} values of 0.52 and 0.62 μM against CV or VV, respectively, compared to CDV EC_{50} values of 42 or 31 μM. ODE-CDV also had significantly lower EC_{50} values of 0.23 or 0.21 μM against CV or VV (15). Although *in vitro* the analogs proved to be more cytotoxic than the parent compound, CDV, their enhanced activity and selectivity indices suggested that these analogues needed to be evaluated for their oral efficacy in animal models of orthopoxvirus disease in view of a recent report that the relative oral bioavailability of HDP-CDV, ODE-CDV and OLP-CDV ranges from 88 to 97% (6) versus <5% for oral cidofovir (25).

The purpose of the studies reported here was to determine the comparative efficacy of parenteral CDV with oral HDP-CDV, ODE-CDV, OLE-CDV or OLP-CDV on lethal CV and VV infections in mice. Since we have reported previously that CDV is highly active in these models when given as single or multiple doses either prior to or after infection, similar studies were carried out with these analogs. In addition, we also determined the effect of oral treatment with HDP-CDV or ODE-CDV on the replication of CV or VV in important target organs.

Materials and Methods

In Vitro Efficacy and Toxicity

The activity and toxicity of the four alkoxyalkyl analogs *in vitro* were determined in human foreskin fibroblast cells using methods described previously (15).

Virus

Cowpox virus, strain Brighton, was kindly provided by John W. Huggins, Ph.D. (U.S. Army Medical Research Institute of Infectious Disease, Frederick, MD.). Vaccinia virus, strain WR, was obtained from American Type Tissue Collection, Washington, DC.

Mice

Female BALB/c mice, 3 weeks of age were obtained commercially (Charles River Laboratories, Raleigh, NC and Wilmington, VA., respectively). Mice were group housed in microisolator cages and utilized at a quantity of 15 mice per treatment group for statistical analysis. Mice were obtained, housed, utilized and euthanized according to USDA and AAALAC regulatory policies. All animal procedures were approved by University of Alabama at Birmingham, Institutional Animal Care and Use Committee prior to initiation of studies.

Antiviral Compounds

Cidofovir (Vistide®, Gilead Pharmaceuticals, Foster City, CA) was diluted in sterile saline to yield the desired dosages within a 0.1 ml volume. It was administered intraperitoneally (i.p.) once daily for 5 days, or 1 to 2 times weekly for periods of 7 days, or as a single dose depending on the experimental protocol. HDP-CDV and ODE-CDV were synthesized, purified and characterized as reported previously (2, 15) and provided as dry powders. OLP-CDV and OLE-CDV were synthesized by the same method using oleyl bromide instead of hexadecyl bromide or octadecyl bromide (2,15). The degree of purity was similar to that reported for HDP-CDV and ODE-CDV. The synthesis, purification and analytical data for these two compounds will be reported elsewhere (W.B. Wan, personal communication, 2003). Structures of the respective compounds is shown in Figure 1.

The dry powders were weighed and dissolved in deionized water to yield the desired dosages within a 0.2 ml volume for oral gavage. Each was administered orally once as a single dose, twice weekly or daily up to 5 days, depending on the experimental protocol. Uninfected mice served as toxicity controls for each compound and were treated similarly.

Experimental Infections And Viral Pathogenesis

Infections were initiated by intranasal inoculation of anesthetized (ketamine-xylazine) BALB/c mice (18). CV-BR (5.3 - 9 x 10^5 pfu/animal) or VV-WR (1 x 10^4 pfu/animal) was instilled using a micropipetor and a total volume of 40 µl per animal. Samples of lung, liver, kidney and spleen were obtained from 3 mice per treatment group as described previously (19) on Days 1, 3, 5, 7, 10, 12 or 15 following CV-BR or VV-WR infections. Samples were homogenized in media (10% wt/vol) and frozen at -70° C until analyzed for virus titer.

Virus Quantitation

Samples were thawed and assayed on Vero cells using an agarose overlay plaque assay to determine CV-BR titers (15, 18). Briefly, samples of organ homogenates were diluted serially and a 0.2 ml volume was placed into each of 12-wells of Vero cell monolayers and incubated 1 h. A 0.5% agar in minimal essential medium (MEM) (SeaKem®, ME agarose, FMC BioProducts, Rockland, MD) solution was added to each well and the cultures were incubated for 3 days. Cultures were stained with neutral red (Gibco, Rockland, MD.) for approximately 6 h prior to enumeration of viral plaques.

Statistical Evaluations

Mortality rates were analyzed by Fisher's exact test and mean day of death and virus titers in tissues using Mann-Whitney U rank sum test. A p value ≤ 0.05 was considered significant.

Results

In vitro activity of ether lipid analogs of CDV

We have previously reported that HDP-CDV and ODE-CDV were about 50-200 fold more active than CDV against CV and VV in HFF cells (15). For comparison purposes, these results along with those for CDV are included with OLE-CDV and OLP-CDV in **Table 1**. The EC_{50} for OLP-CDV was 0.4 μM for CV and VV, whereas the two viruses were inhibited by 50% by only 0.06 and 0.07 μM of OLE-CDV, respectively. All four of the analogs were approximately 50 – 500 fold more active against CV and VV than unmodified CDV.

Activity of HDP-CDV, ODE-CDV, OLP-CDV or OLE-CDV in CV infections of mice

Compounds were prepared in deionized water at 20, 6.7 or 2 mg/kg dosages and administered once daily for 5 consecutive days by oral gavage to infected mice beginning 24, 48 or 72 h post viral inoculation. CDV was administered i.p. in similar doses for comparison, although it should be noted that the molecular weight of CDV is roughly 50% of that of the ether lipid conjugates of CDV. Toxicity was associated with the 20 mg/kg dose of HDP-CDV, ODE-CDV, OLP-CDV or OLE-CDV and some mortality and weight loss was observed (data not shown). At the nontoxic 6.7 mg/kg dose, each compound significantly reduced final mortality ($p \leq 0.01$) at one or more time points (**Table 2**). Both HDP-CDV and ODE-CDV significantly reduced mortality rates when treatment was initiated as late as 72 h post viral inoculation. Treatment with the 2 mg/kg dose with all four analogs was ineffective. Treatment with CDV resulted in significant protection from mortality with all dosages at all time points with the exception of the lowest dose administered, 2 mg/kg, at 48 or 72 h (data not shown).

Activity of HDP-CDV, ODE-CDV, OLP-CDV or ODE-CDV in VV infections of mice

Compounds were prepared in deionized water at dosages of 10, 5 or 2.5 mg/kg and administered once daily for five consecutive days by oral gavage to infected mice beginning 24, 48 or 72 hr post viral inoculation. CDV was administered i.p. in similar doses for comparison. At 5 mg/kg, HDP-CDV, ODE-CDV or OLE-CDV significantly reduced final mortality rates ($p \leq 0.01$) at 24 or 48 hr post inoculation (**Table 3**). None of the compounds exhibited activity when treatments were initiated 72 hr post inoculation. The positive control, CDV, was effective at

all dosages and all time points with the exception of the lowest dosage administered, 2.5 mg/kg, given at 72 hr post inoculation (data not shown).

Effect of Pretreatment with HDP-CDV or ODE-CDV on CV infections of mice

We have reported previously that CDV can protect mice infected with CV or VV when given as early as 5 days prior to infection (18). HDP-CDV and ODE-CDV were also evaluated for their prophylactic activity by treating mice by oral gavage with 10 or 5 mg/kg or 5 or 2.5 mg/kg, respectively, beginning 5, 3 or 1 day prior to viral inoculation. For comparison, CDV was given i.p. at 10 or 5 mg/kg. Groups treated beginning 5 days prior to infection were dosed once daily for 5 consecutive days up to Day 0 which was the day of viral inoculation. Groups beginning 3 days prior to infection received a total of 3 daily doses prior to the day of infection and groups beginning 1 day received only a single dose 24 hours prior to viral inoculation. The results in **Table 4** indicated that HDP-CDV and ODE-CDV, as well as, CDV, were highly protective against mortality due to CV infection when given one to five days prior to infection.

Effect of Single Dose Treatment with HDP-CDV or ODE-CDV on CV-BR Infections of mice

CDV has also been reported by us and others to significantly reduce mortality of CV or VV infected mice with only one or two doses due to the long intracellular half-life of this drug (3, 18, 20). In a similar study, we gave HDP-CDV as a single dose of 25 or 12.5 mg/kg or ODE-CDV 20 or 10 mg/kg on Days -5, -3, -1, +1 or +3 prior to or after i.n. CV inoculation and all regimens used provided significant protection from mortality at all time points with both compounds (**Table 5**). In addition, CDV at 30 mg/kg given as a single dose i.p. was also protective.

Effect of HDP-CDV or ODE-CDV on the Pathogenesis of CV-BR and VV-WR Infections of mice

To determine the effect of treatment with HDP-CDV or ODE-CDV on the replication of CV in target organs of mice, animals were inoculated with CV and treated orally with 5 mg/kg of HDP-CDV or ODE-CDV once daily for 5 days beginning 24 h after infection. On various days post infection, animals were euthanized, tissues removed and assayed for CV. All of the treatment regimens resulted in a significant reduction in mortality, and a 3-5 $\log_{10}$ decrease in virus titers in liver, spleen and kidney. However, there were no alterations in virus titers in lung. Similar results were observed with CDV given i.p. (**Figure 2**).

In order to determine the effect of these compounds on replication of VV in tissues, HDP-CDV or ODE-CDV was given at 5 mg/kg once daily for 5 days beginning 24 hours post viral inoculation. Both HDP-CDV and ODE-CDV reduced viral replication by ≥ 2 logs in liver, spleen and kidney of VV infected mice (**Figure 3**). As with CV, lung titers remained unaffected although all treated mice survived.

Since CV is only moderately sensitive to the action of CDV, we postulated that higher doses of the drugs might be necessary to reduce viral titers in the lung. The levels of HDP-CDV and ODE-CDV in lung are 43 and 28 times lower than those found in liver and 5.8 and 4.8 times lower than found in kidney where antiviral effects were demonstrated (Ciesla, 2003). To test this hypothesis, we utilized higher doses of HDP-CDV or ODE-CDV given twice weekly and determined the effect on viral titers in the same target organs. The results of this experiment are shown in **Figure 4**. When HDP-CDV or ODE-CDV was given at 30 mg/kg on day 1 and 5 post viral inoculation, treatment reduced viral replication by ≥ 2.5 $\log_{10}$ in liver, spleen and kidney of CV infected mice. There was a 1-2 $\log_{10}$ reduction in CV titers in lung tissue on Days 3 and 5 after treatment with HDP-CDV or ODE-CDV at 30 mg/kg which was suggestive of a small dose response and indicated a correlation between drug levels in lung and effect on viral replication.

Discussion

Cidofovir has been reported to be highly effective against orthopoxvirus infection *in vitro* and in murine models of cowpox and vaccinia virus infections (3, 4, 8, 9, 15, 18, 20-24) and is currently the drug of choice for treatment of potential outbreaks of smallpox, monkeypox, and vaccination complications. Although it has good activity in experimental systems, its usefulness in humans is limited by its nephrotoxicity and lack of oral activity. A number of approaches have been taken to enhance the oral activity nucleosides and nucleotides, including the synthesis of prodrugs, as was done for acyclovir (Valtrex®), ganciclovir (Valcyte®) penciclovir (Famvir®), tenofovir (Viread®) and adefovir (Preveon®), or conjugation to various lipid molecules to facilitate oral absorption (11-13) or the current report regarding synthesis of ether lipid esters of current antiviral agents. We found previously that alkylglycerol phosphate or alkoxypropyl phosphate esters of acyclovir and ganciclovir had greater oral bioavailability in rodents and were active orally in animal models of herpes simplex virus, murine cytomegalovirus and woodchuck hepatitis virus infections (12, 13).

To obtain better oral bioavailability with CDV, similar methodology was utilized to produce HDP-CDV or HDP-cCDV (2, 15). A comparison of the activity against vaccinia and cowpox viruses between the parent nucleotides, CDV, cCDV and the alkoxyalkyl ester analogs indicated that while it required 25 – 50 μM of CDV or cCDV to inhibit the replication of the four

orthopoxviruses tested, while HDP-CDV and HDP-cCDV analogs were active at levels 50- to 200-fold less than the parent molecules (15). Although cytotoxicity of the analogs was increased, the selectivity index of HDP-CDV or HDP-cCDV was increased considerably over that of the parent compounds (15). HDP-CDV, ODE-CDV and OLP-CDV are highly absorbed when given orally to mice resulting in plasma levels which would be well above inhibitory levels required for *in vitro* replication of orthopoxviruses (6). It was further demonstrated that the analog HDP-[2-^{14}C]CDV has a greater than 11-23 fold increase in cellular uptake over that of -[2-^{14}C]CDV and that the intracellular levels of CDV-diphosphate, the active antiviral agent, were 100 times greater than observed with CDV (1). This enhanced uptake and conversion to the antiviral diphosphate appear to be responsible for the significant enhancement in activity seen with both orthopoxviruses (15) and herpesvirus infections *in vitro* (2).

As part of the current studies, two additional ether lipid conjugates, OLE-CDV and OLP-CDV were compared with CDV *in vitro* and both were more active than either HDP-CDV or ODE-CDV. In animal studies, oral bioavailability and antiviral efficacy of all 4 analogs was demonstrated. The antiviral activity of HDP-CDV, ODE-CDV or OLE-CDV against CV and VV was effective at nontoxic dosages and treatment could be delayed until 48-72 h post infection and still alter mortality rates. Based on the initial experiments evaluating the 4 compounds against mortality due to CV or VV, and the PK profile of HDP-CDV, the analogs HDP-CDV and ODE-CDV were selected for further evaluations. In all experiments, both of the orally active analogs were at least as effective in reducing mortality and viral replication in target organs as parenteral CDV. In addition, both were active either prophylactically or therapeutically as previously described with CDV (18).

It is interesting that even though HDP-CDV and ODE-CDV were significantly more active than CDV against both viruses *in vitro*, they were equally effective in reducing viral replication in the liver, spleen and kidney, but no more effective than CDV in reducing replication in the lung. In contrast to levels of compound with oral HDP-CDV, drug levels in lung are much lower after parenteral administration of CDV and the majority of drug is found in kidneys with significant amounts in liver (6). Conversely, HDP-CDV and ODE-CDV when given orally are present at much higher levels in liver than the kidney and should maintain therapeutic tissue levels with less nephrotoxicity. In a study by Bray et. al., CDV given subcutaneously at 100 mg/kg significantly reduced virus titers in lung (4). When Smee et. al. administered CDV directly to the site of inoculation via aerosol or intranasal instillation, lung titers were also significantly reduced (22). It should be pointed out, however, that in these murine models infection of the lung may not be a critical part of the pathogenesis as treated animals do

survive with high levels of virus in the lung. Finally, although the C_{max} levels of total drug were higher in lung with oral HDP-CDV and ODE-CDV than with parenteral CDV, 1.2 versus 0.4 nmol/gm, (6), the levels of the key metabolite, CDV-diphosphate, have not been measured in lung tissue.

A correlation between *in vitro* and *in vivo* efficacy is further demonstrated by the observation that murine CMV is greater than 100-fold more sensitive to CDV and 40,000 fold more sensitive to the CDV analogs than CV and VV (2, 15). Treatment of MCMV infections with CDV, HDP-CDV or ODE-CDV is highly effective in reducing viral replication in all tissues including the lungs of treated animals (Kern, E.R., Hartline, C., Bidanset, D., Quenelle, D.C., Beadle, J.R., and Hostetler, K.Y. 2003. Enhanced activity of orally active alkoxyalkyl esters of cidofovir against experimental cytomegalovirus infections. Abstracts of 9th International Cytomegalovirus Workshop, Abstract H.05, May 20-25, Maastricht, The Netherlands). Therefore it appears that *in vitro* sensitivity and blood and tissue levels of drug are important determinates of antiviral efficacy and may be variable in different tissues depending on the uptake and conversion of the drug to the active metabolite.

HDP-CDV and ODE-CDV are both orally bioavailable, have high antiviral efficacy, persist in tissues for a relatively long period of time (6). The present results comparing i.p. CDV and oral HDP-CDV and ODE-CDV show that these compounds when given orally and are at least equivalent to CDV given parenterally. Our results are consistent with pharmacokinetic data indicating that oral bioavailability and persistence in tissues of up to 72 hours after oral administration of HDP-CDV, ODE-CDV and OLP-CDV and persistence of therapeutic drug levels in critical organs of lung, liver, kidney and skin after a single oral dose of 10 mg/kg. This translates into oral dosing once or twice weekly instead of daily dosing. Since kidney exposure is reported to be low with oral HDP-CDV or ODE-CDV, one would anticipate reduced nephrotoxic adverse events. These orally active compounds, HDP-CDV and ODE-CDV, are at least equivalent to i.p. CDV in these studies and should be effective when used for prophylaxis, post-exposure prophylaxis or treatment for smallpox and other orthopoxviruses including monkeypox which has become a problem due to unexpected outbreaks and increasing incidences of natural transmission (5, 10).

Acknowledgements

This work was supported by Public Health Service Contract Number NO1-AI-65290, from NIAID, NIH, Bethesda, MD, and by NIH grants EY11832, AI29164 and by the Department of

the Army, Grant no. 17-01-2-0071. The U.S. Army Medical Research Acquisition Activity, Ft. Detrick, MD, is the awarding acquisition office. The content of this article does not necessarily reflect the position or policy of the government, and no official endorsement should be inferred. Excellent technical assistance for in vitro analyses was kindly provided by Ms. Kathy A. Keith and in vivo by Ms. Bridgett P. Herrod.

References

1) Aldern, K.A., S. Ciesla, K.L. Winegarden, and K.Y. Hostetler. 2003. Increased antiviral activity of 1-O-hexacecyloxypropyl-[2-^{14}C] cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism, Mol. Pharmacol. **63**, 678-681.

2) Beadle, J.R., C. Hartline, K.A. Aldern, N. Rodriguez, E. Harden, E.R. Kern, and K.Y. Hostetler. 2002. Alkoxyalkyl esters of cidofovir exhibit multiple-log enhancement of antiviral activity against cytomegalovirus and herpesvirus replication *in vitro*, Antimicrob. Agents Chemother. **46**, 2381-2386.

3) Bray, M.,M. Martinez,, D.F. Smee, D. Kefauver, E. Thompson, and J.W.Huggins. 2000. Cidofovir protects mice against lethal aerosol or intranasal cowpox virus challenge, J. Infect. Dis. **181**, 10-19.

4) Bray, M., M. Martinez,D. Kefauver,M. West, and C.Roy. 2002. Treatment of aerosolized cowpox virus infection in mice with aerosolized cidofovir, Antiviral Res. **54,** 129-142.

5) Centers for Disease Control and Prevention. 2003. Multistate outbreak of monkeypox - - - Illinois, Indiana, and Wisconsin, 2003. MMWR Morbid and Mortal Wkly Rep, 52(23); 537-540.

6) Ciesla, S.L., J. Trahan, W.B. Wan, J.R. Beadle, K.A. Aldern, G.R. Painter and K.Y. Hostetler, 2003. Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney, Antiviral Res., in press.

7) Cundy, K.C., Bidgood, A.M., Lynch, G., Shaw, J.P., Griffin, L., and Lee, W.A. 1996. Pharmacokinetics, bioavailability, metabolism, and tissue distribution of cidofovir (HPMPC) and cyclic HPMPC in rats. Drug Metab. Dispos. **24**, 745-752.

8) De Clercq, E., T. Sakuma, M. Baba, R Pauwels, J. Balzarini, I. Rosenberg, I. and A. Holy. 1987. Antiviral activity of phosphonylmethoxyalkyl derivatives of purine and pyrimidines, Antiviral Research 8:261-272.

9) De Clercq, E. and J. Neyts, 1993. Efficacy of (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine for the treatment of lethal vaccinia virus infections in severe combined immune deficiency (scid) mice, J. Med. Virol., 41:242-246.

10) Heymann, D. L., M. Szczeniowski, and K. Esteves. 1988. Re-emergence of monkeypox in Africa: a review of the past six years. Br. Med. Bull. **54,** 693-702.

11) Hostetler, K.Y., Beadle, J.R., Kini, G.D., Gardner, M.F., Wright, K.N., Wu, T.-H., Korba, B.E. 1997. Enhanced oral absorption and antiviral activity of 1-*O*-octadecyl-sn-glycero-

3-phospho-acyclovir and related compounds in hepatitis B virus infection, *in vitro*. Biochem. Pharmacol. 53, 1815-1822.

12) Hostetler, K.Y., Beadle, J.R., Hornbuckle, W.E. Bellezza, C.A., Tochkov, I.A., Cote, P.J., Gerin, J.L. Korba, B.E. and Tennant, B.C. 2000. Antiviral activities of oral 1-O-hexadecylpropanediol-3-phospho-ganciclovir and acyclovir in woodchucks with chronic woodchuck hepatitis virus infection. Antimicrob. Agents Chemother. 44, 1964-1969.

13) Hostetler, K.Y., R.J. Rybak, J.R. Beadle, M.F. Gardner, K.A. Aldern, K.N. Wright, and E.R. Kern. 2001. *In vitro* and *in vivo* activity of 1-O-hexadecylpropanediol-3-phospho-ganciclovir and 1-O-hexadecylpropanediol-3-phospho-penciclovir in cytomegalovirus and herpes simplex virus infections. Antivir. Chem. Chemother. **12,** 61-70.

14) Jacobson, M.A., S. Wilson, H. Stanley,C. Holtzer, J. Cherrington, and S. Safrin. 1999. Phase I study of combination therapy with intravenous cidofovir and oral gangciclovir for cytomegalovirus retinitis in patients with AIDS, Clin. Infect. Dis. **28**, 528-533.

15) Kern, E.R., C. Hartline, E. Harden, K. Keith, N. Rodriguez, J.R. Beadle, and K.Y. Hostetler. 2002. Enhanced inhibition of orthopoxvirus replication *in vitro* by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob. Agents Chemother. **46**, 991-995.

16) LeDuc, J.W., I. Damon, D.A. Relman, J. Huggins, and P.B. Jahrling. 2002. Smallpox research activities: U.S. interagency collaboration, 2001, Emerg. Infect. Dis. **8**, 743-745.

17) LeDuc, J.W. and P.B. Jahrling. 2001. Strengthening national preparedness for smallpox: an update, Emerg. Infect. Dis. **7**, 155-157.

18) Quenelle,D.C., D.J. Collins,and E.R. Kern. 2003. Efficacy of multiple and single dose cidofovir in vaccinia and cowpox virus infections of mice. Antimicrob. Agents Chemother. (AAC, in press, 2003).

19) Rybak, R.J., J. Zemlika, Y. Qiu, C.B. Hartline, and E.R. Kern. 1999. Effective treatment of murine cytomegalovirus infections with methlenecyclopropane analogues of nucleosides, Antiviral Res. **43**, 175-188.

20) Smee, D.F., K.W. Bailey and R.W. Sidwell. 2001. Treatment of lethal vaccinia virus respiratory infections in mice with cidofovir, Antivir. Chem. Chemother. **12**, 71-76.

21) Smee, D.F., K.W. Bailey, and R.W. Sidwell. 2000. Treatment of cowpox virus respiratory infections in mice with ribavirin as a single agent or followed sequentially by cidofovir, Antiviral Chem. Chemother. **11**, 303-309.

22) Smee, D.F., K.W. Bailey, M.H. Wong, and R.W. Sidwell. 2000 Intranasal treatment of cowpox virus respiratory infections in mice with cidofovir, Antiviral Res. **47**, 171-177.

23) Smee, D.F., K.W. Bailey, M.H. Wong, and R.W. Sidwell. 2001. Effects of cidfovir on the pathogenesis of a lethal vaccinia virus respiratory infection in mice, Antiviral Res. **52,** 55-62.

24) Snoeck, R., A. Holý, C. Dewolf-Peeters, J. Van Den Oord, E. DeClercq, and G. Andrei. 2002. Antivaccinia activities of acyclic nucleoside phosphonate derivatives in epithelial cells and organotypic cultures. Antimicrob. Agents Chemother. **46**, 3356-3361.

25) Wachsman M, B.G.Petty K.C. Cundy, H.S. Jaffe, P.E. Fisher, A. Pastelak and P.S. Lietman. 1996. Pharmacokinetics, safety and bioavailability of HPMPC (cidofovir) in human immunodeficiency virus-infected subjects, Antiviral Res., 29:153-61.

Table 1: Efficacy and Cytotoxicity of Alkoxyalkyl Esters of CDV

Compound	Vaccinia Copenhagen			Cowpox Brighton		
	EC_{50}[a] (μM)	CC_{50}[a] (μM)	Selectivity Index[b]	EC_{50}[a] (μM)	CC_{50}[a] (μM)	Selectivity Index[b]
CDV	31±5.4	>317±0	>10	42±5.4	>317±0	>7.5
OLP-CDV	0.4±0.2	87±15	218	0.6±0.3	87±15	145
OLE-CDV	0.06±0.02	56±29	933	0.07±0.02	56±29	800
HDP-CDV[c]	0.8±0.4	31±24	37	0.6±0.3	31±24	53
ODE-CDV[c]	0.2±0.1	14	65	0.3±0.3	14	49

a. Values are the mean of 2 or more assays ± standard deviation

b. Selectivity Index (SI) = CC_{50} /EC_{50}.

c. From Kern et al AAC, 2002, 46:991-995.

Table 2: Effect of Oral Treatment with HDP-CDV, ODE-CDV, OLE-CDV, or OLP-CDV on Mortality of BALB/c Mice Inoculated Intranasally with Cowpox-BR Virus

	Mortality				
Treatment[a]	Number	Percent	P-value	MDD[b]	P-value
Placebo					
Saline +24 h	15/15	100	---	9.7	---
CDV					
6.7 mg/kg +24 h	0/15	0	<0.001	---	---
6.7 mg/kg +48 h	0/15	0	<0.001	---	---
6.7 mg/kg +72 h	5/15	33	<0.001	13.2	<0.01
Placebo					
Water +24 h	15/15	100	---	9.3	---
HDP-CDV					
6.7 mg/kg +24 h	6/15	40	0.001	9.5	NS[c]
6.7 mg/kg +48 h	12/14	86	NS	10.5	NS
6.7 mg/kg +72 h	7/15	47	<0.01	12.7	<0.001
ODE-CDV					
6.7 mg/kg +24 h	3/13	23	<0.001	9.3	NS
6.7 mg/kg +48 h	6/14	43	<0.01	12.7	0.01
6.7 mg/kg +72 h	7/13	54	0.02	11.6	0.07
OLP-CDV					
6.7 mg/kg +24 h	12/14	86	NS	11.4	<0.01
6.7 mg/kg +48 h	4/14	29	<0.001	12.5	0.09
6.7 mg/kg +72 h	12/14	86	NS	10.3	0.02
OLE-CDV					
6.7 mg/kg +24 h	8/15	53	<0.01	13.0	NS
6.7 mg/kg +48 h	5/15	33	<0.001	12.0	<0.001
6.7 mg/kg +72 h	11/14	79	NS	11.5	0.02

a. Test compounds were prepared daily in water and delivered orally in 0.2 ml doses except CDV which was prepared in sterile saline and delivered ip. in 0.1 ml doses. Animals were treated once daily for 5 days beginning 24, 48 or 72 h post viral inoculation.
b. MDD = Mean Day of Death.
c. NS = Not significant when compared to the placebo control.

Table 3: Effect of Oral Treatment with HDP-CDV, ODE-CDV, OLE-CDV, or OLP-CDV on Mortality of BALB/c Mice Inoculated Intranasally with Vaccinia-WR Virus

	Mortality				
Treatment[a]	Number	Percent	P-value	MDD[b]	P-value
Placebo					
Saline +24 h	15/15	100	---	6.8	---
CDV					
5 mg/kg +24 h	0/15	0	<0.001	---	---
5 mg/kg +48 h	4/15	27	<0.001	7.8	0.01
5 mg/kg +72 h	0/15	0	<0.001	---	---
Placebo					
Water +24 h	15/15	100	---	6.8	---
HDP-CDV					
5 mg/kg +24 h	2/14	13	<0.001	11.0	<0.05
5 mg/kg + 48 h	10/15	67	<0.05	8.0	<0.01
5 mg/kg + 72 h	14/15	93	NS[c]	7.4	0.07
ODE-CDV					
5 mg/kg +24 h	0/15	0	<0.001	---	---
5 mg/kg +48 h	6/15	40	0.001	8.0	0.06
5 mg/kg +72 h	15/15	100	NS	7.3	<0.01
OLP-CDV					
5 mg/kg +24 h	11/15	73	NS	9.6	<0.001
5 mg/kg +48 h	12/15	80	NS	7.3	<0.01
5 mg/kg +72 h	15/15	100	NS	7.0	<0.05
OLE-CDV					
5 mg/kg +24 h	4/15	27	<0.001	7.5	NS
5 mg/kg +48 h	9/15	60	<0.05	7.4	<0.01
5 mg/kg +72 h	14/14	100	NS	6.5	NS

a.. Test compounds were prepared in water and delivered orally in 0.2 ml doses. CDV was prepared in sterile saline and was delivered ip. in 0.1 ml doses. Animals were treated once daily for 5 days beginning 24, 48 or 72 h post viral inoculation.

b. MDD = Mean Day of Death.

c. NS = Not significant when compared to the placebo control.

Table 4: Effect of Pre-Infection Oral Treatments with HDP-CDV or ODE-CDV on Mortality of BALB/c Mice Inoculated Intranasally with Cowpox-BR Virus

Treatment[a]	Mortality Number	Percent	P-value	MDD[b]	P-value
Placebo					
Deionized H20 Day-5	15/15	100	---	10.1	---
Day -5					
CDV 10 mg/kg	1/15	7	<0.001	12.0	0.09
CDV 5 mg/kg	1/15	7	<0.001	17.0	0.07
HDP 10 mg/kg	0/15	0	<0.001	---	---
HDP 5 mg/kg	0/15	0	<0.001	---	---
ODE 5 mg/kg	3/15	20	<0.001	5.7	NS[c]
ODE 2.5 mg/kg	5/13	38	<0.001	8.6	NS
Day -3					
CDV 10 mg/kg	2/15	13	<0.001	9.0	NS
CDV 5 mg/kg	6/15	40	<0.01	11.0	0.05
HDP 10 mg/kg	5/14	36	<0.001	9.6	NS
HDP 5 mg/kg	3/13	23	<0.001	15.0	<0.01
ODE 5 mg/kg	2/15	13	<0.001	11.0	0.07
ODE 2.5 mg/kg	1/15	7	<0.001	13.0	0.07
Day -1					
CDV 10 mg/kg	1/15	7	<0.001	12.0	0.09
CDV 5 mg/kg	5/15	33	<0.001	11.4	0.06
HDP 10 mg/kg	4/14	29	<0.001	9.3	NS
HDP 5 mg/kg	6/15	40	<0.01	10.7	0.06
ODE 5 mg/kg	0/14	0	<0.001	---	---
ODE 2.5 mg/kg	4/15	27	<0.001	11.5	<0.05

a CDV was prepared in sterile saline and delivered ip. in 0.1 ml doses. HDP-CDV and ODE-CDV were prepared in deionized water and delivered p.o. in 0.2 ml doses. Animals were treated once daily beginning Day -5, -3, or -1 through Day 0, or viral inoculation day.

b MDD = Mean Day of Death.

c NS = Not significant when compared to the placebo control.

Figure 4: Effect of Oral Treatment with HDP-CDV or ODE-CDV Given Twice Weekly on the Pathogenesis of CV-BR Infections in Mice

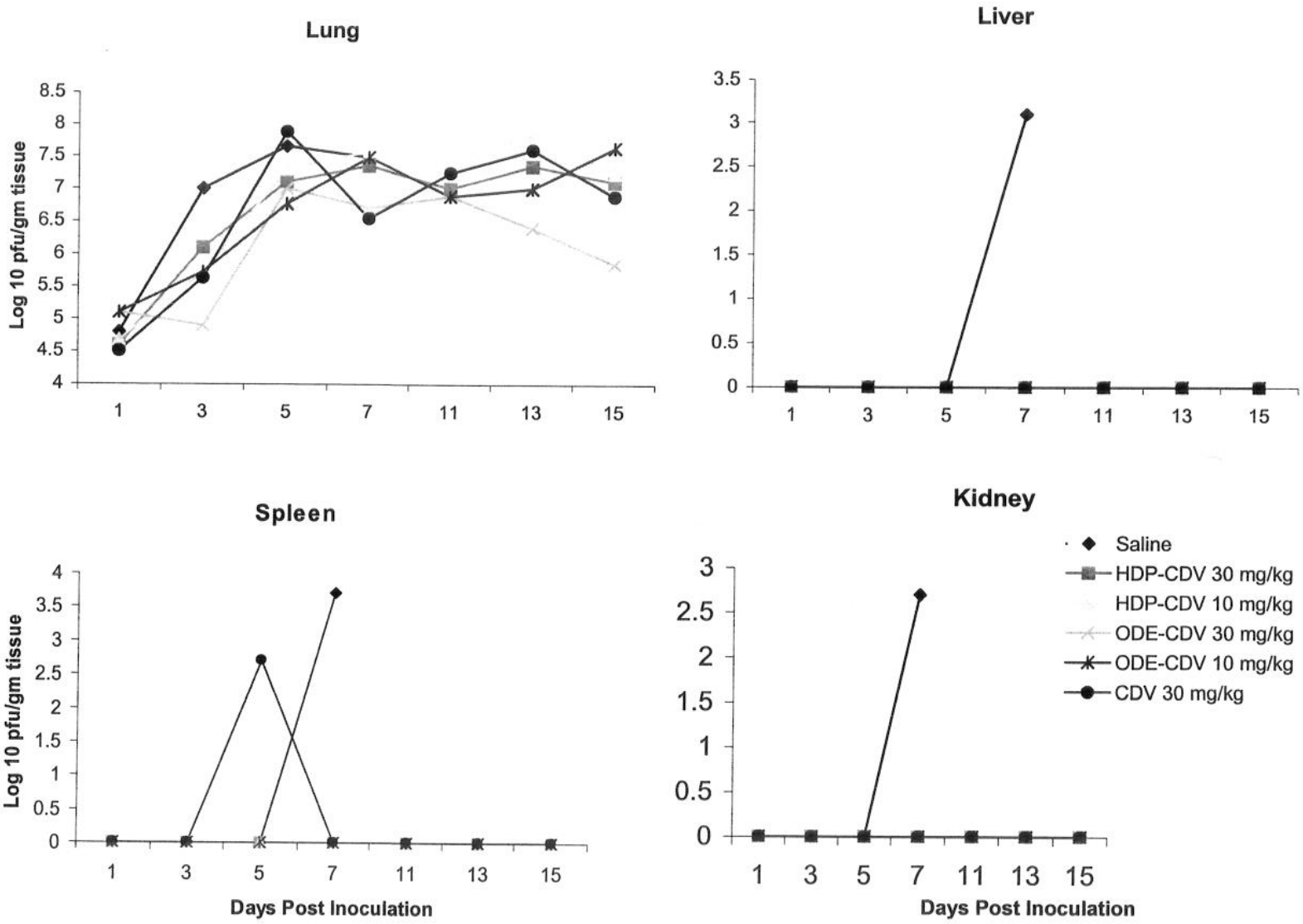


Figure 2: Effect of Daily Oral Treatment with HDP-CDV or ODE-CDV on the Pathogenesis of CV-BR Infections in Mice

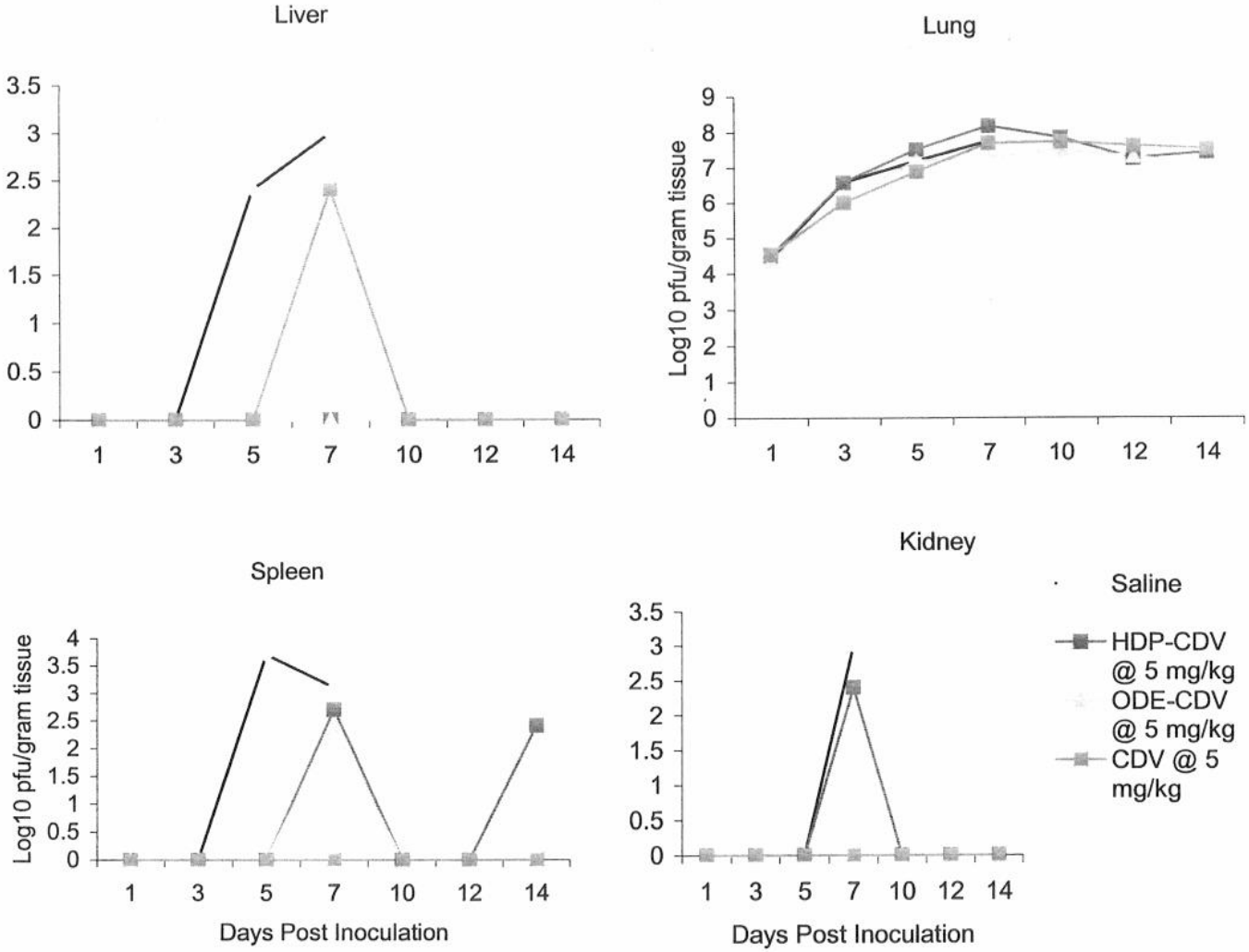

Liver
3.5
3
2.5
2
1.5
1
0.5
0
1 3 5 7 10 12 14
Lung
Log10 pfu/gram tissue
9
8
7
6
5
4
3
2
1
0
1 3 5 7 10 12 14
Spleen
Log10 pfu/gram tissue
4
3.5
3
2.5
2
1.5
1
0.5
0
1 3 5 7 10 12 14
Days Post Inoculation
Kidney
3.5
3
2.5
2
1.5
1
0.5
0
1 3 5 7 10 12 14
Days Post Inoculation
Saline
HDP-CDV @ 5 mg/kg
ODE-CDV @ 5 mg/kg
CDV @ 5 mg/kg

Figure 3: Effect of Daily Oral Treatment with HDP-CDV or ODE-CDV on the Pathogenesis of VV-WR Infections in Mice

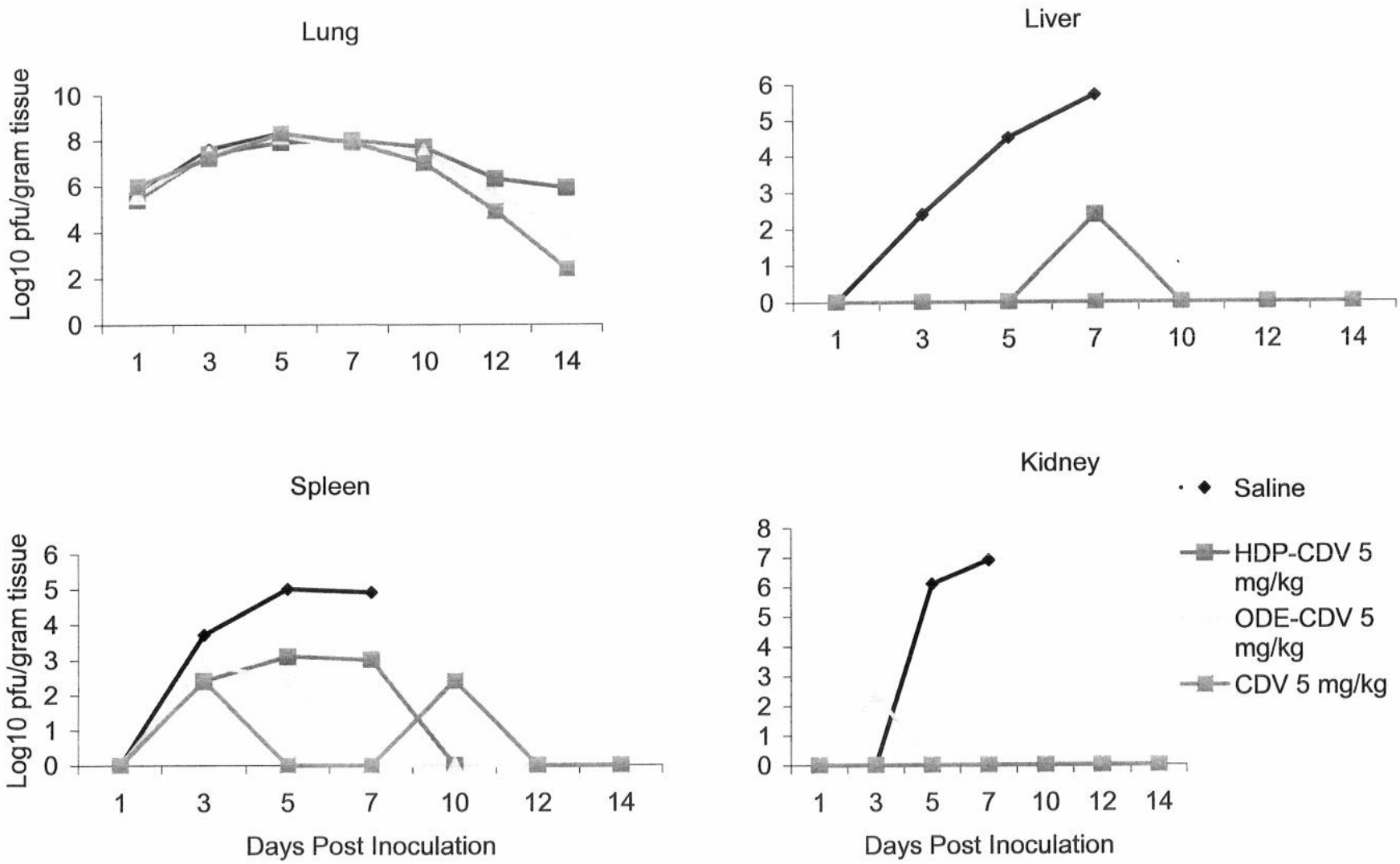
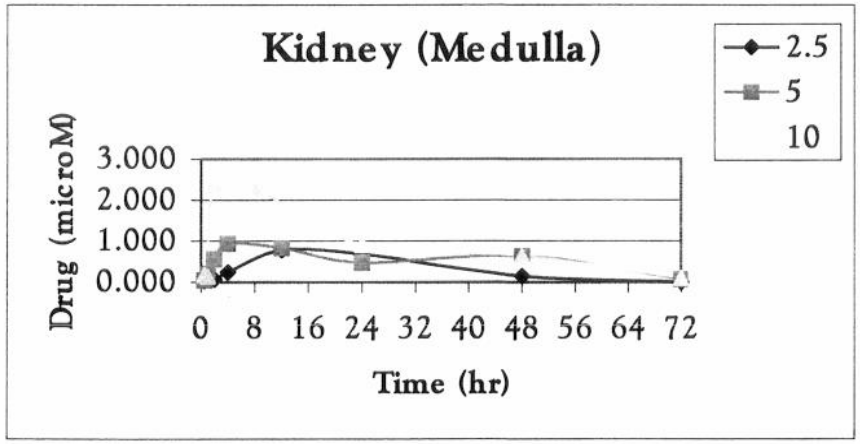
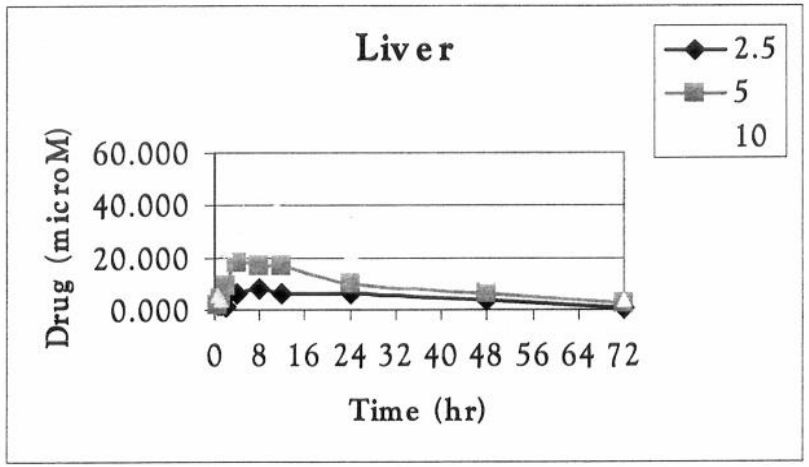
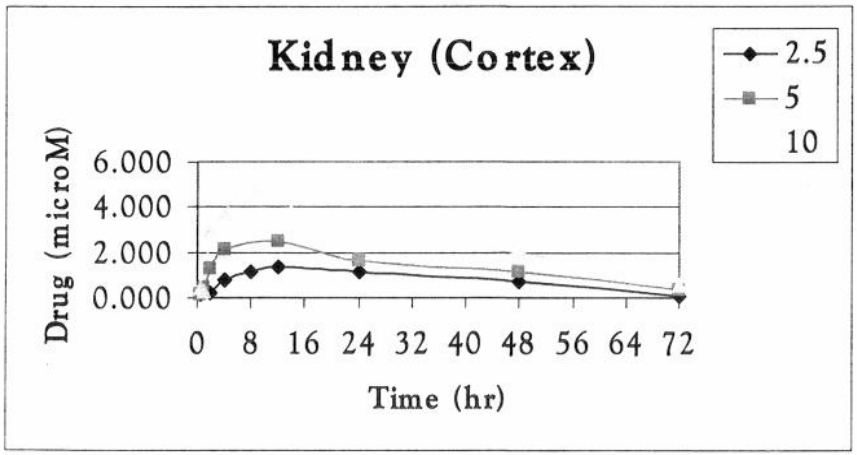
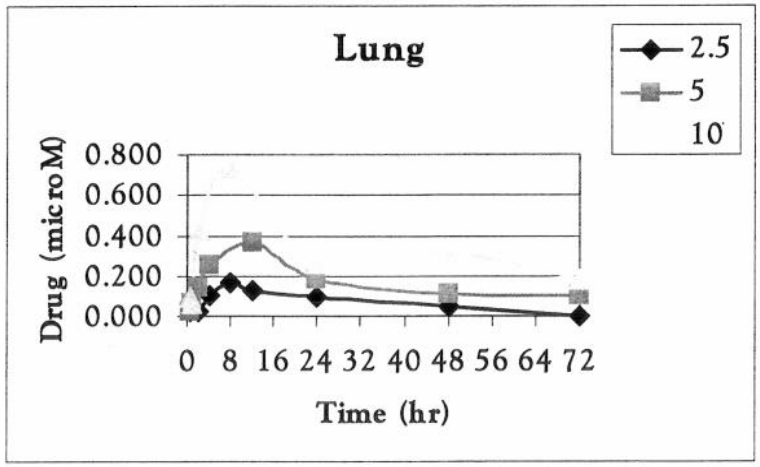


Figure 1: Pharmacokinetic profile of ^{14}C-HDP-CDV following a single oral dose of 10, 5 or 2.5 mg/kg









Abstract

Cidofovir (CDV) is a highly effective inhibitor of orthopoxvirus replication, and may be used intravenously to treat smallpox or complications arising from the smallpox vaccine under an investigational new drug application (IND). However, CDV is absorbed poorly following oral administration and is inactive orally. To improve the bio-availability of CDV, others synthesized alkoxyalkanol esters of CDV and observed >100 fold more activity than unmodified CDV against cowpox, vaccinia, and variola virus replication. These ether lipid analogs of CDV have high oral bioavailablity in mice. In this study, we compared the oral activity of CDV with the hexadecyloxpropyl-, octadecyloxyethyl-, oleyloxypropyl- and oleyloxyethyl- esters of CDV in a lethal, aerosol ectromelia virus challenge model in A/NCR mice. Octadecyloxyethyl-CDV appeared to be the most potent CDV analog as a dose regimen of 5mg/kg started 4 hours following challenge completely blocked virus replication in spleen and liver, and protected 100 % of A/NCR mice, while oral, unmodified CDV was inactive. These results suggest this family of compounds deserves further evaluation as poxvirus antivirals.

Introduction

With the heightened potential for bioterrorism there is a need for an efficacious antiviral for the treatment of smallpox or human monkeypox, and the complications that arise from the smallpox vaccine. Cidofovir (CDV) is effective against a wide-range of DNA virus and retrovirus infections probably by interfering with viral DNA synthesis (De Clercq, 1998). CDV was first shown active *in vitro* against vaccinia virus (VACV) by De Clercq and coworkers (De Clercq et al., 1987), while others demonstrated 50% effective concentration (EC_{50}) values in the 10-50 μM range against a number of other orthopoxviruses including variola virus (VARV), monkeypox virus and cowpox virus (CPXV) (Kern et al., 2003). Furthermore, parenterally administered CDV was active against lethal VACV and CPXV infections in mice (Neyts & De Clercq, 1993;Bray et al., 2000;Smee et al., 2001), but was poorly absorbed when administered orally (Wachsman et al., 1996). Oral bioavailability is a desirable property of drugs proposed for emergency use in an outbreak of smallpox or monkeypox. Previous studies by Hostetler *et al* showed that derivatization of acyclovir with 1-O-hexadecyl-glycero-3-phosphate provided a compound which was 100% orally bioavailable in mice (Hostetler et al., 1997). Using this approach several ether lipid esters of CDV and cyclic CDV were synthesized and tested *in vitro* against VACV and CPXV (Kern et al., 2002). The hexa-decyloxypropyl- (HDP) and octa-decyloxyethyl- (ODE) esters of CDV had EC_{50} values of 0.2-1.1 and 0.1-0.4 μM, respectively, against five strains of VACV versus 10.1-46.2 μM for CDV; however, no *in vivo* experiments have yet been reported. In this study, we evaluate the antiviral activity of this class of compounds *in vitro* and *in vivo* against a natural mouse pathogen, ectromelia virus (ECTV), the causative agent of mousepox (Buller & Palumbo, 1991). Natural infections with ECTV occur through microscopic abrasions, which allow access of the virus to the epidermal or dermal layers of the cornified mouse skin. ECTV replicates locally at the site of infection, and reaches internal organs via the afferent lymphatics, draining lymph node, and bloodstream (primary viremia). Extensive replication in major organs especially spleen and liver results in detection of virus in blood by 4 or 5 days p.i. (secondary viremia). Virus replication in skin produces the typical exanthem, which can be seen as

early as 6 days p.i., and is dependent on the mouse strain. Virus is transmitted from the primary and secondary lesions. With the A/NCR strain of mouse used in this study, death is observed 7 to 10 days p.i. prior to the development of an exanthem, and is a consequence of extensive liver necrosis. The mousepox model differs from smallpox in the short course of disease, and severe pulmonary pneumonia associated with local replication following an aerosol infection. The major advantage of using ECTV for the evaluation of antiviral compounds lies in its severe disease course in adult, immunocompetent mice, which results in uniform mortality and LD_{50} values of less 1 PFU by the footpad route of infection (Buller & Palumbo, 1991; Chen et al., 1992).

Results

In vitro sensitivity of ectromelia virus to alkoxyalkyl esters of cidofovir

A series of analogs of CDV were synthesized having alkoxyalkanols of varying structure as shown in Fig. 1. (Beadle et al., 2002). As a first step in testing these compounds in an ECTV mouse model, we determined their EC_{50} values in a CV-1 cell plaque reduction assay with VACV strain WR (VACV-WR), ECTV, and a second pool of ECTV prepared with an ECTV recombinant expressing murine IL-4. VACV-WR was included as its sensitivity to CDV and analogs had previously been reported. The EC_{50} for HDP-CDV, ODE-CDV, oleyloxyethyl-CDV (OLE-CDV) and oleyloxypropyl-CDV (OLP-CDV) against the two distinct pools of ECTV were 0.2 to 1.1 μM, which represented a 11 and 60-fold enhanced antiviral activity as compared to CDV (Table 1). The tested compounds had similar EC_{50} values against ECTV and VACV-WR.

Because ECTV has a LD_{50} of less than 1 PFU in the A/NCR strain of mouse following footpad inoculation, this strain was chosen to evaluate the efficacy of CDV analogs (Chen et al., 1992). Preliminary experiments demonstrated that adult A/NCR mice were also highly sensitive by the aerosol route. Doses of 20,000, 1,000, 63, and 6 PFU per mouse gave mean time to death (MTD) values of 8.1, 9.3, 10.2 and 14 days, respectively. The calculated LD_{50} value was 32 PFU. For comparison, the commonly used CPXV mouse model required an aerosol exposure dose of 5×10^6 PFU of CPXV to obtain 100% mortality with ~ 3 week old BALB/c mice, and lowering the exposure dose to 5×10^4 PFU caused only transient, mild illness and weight loss, but no deaths (Bray et al., 2000). Thus the ECTV aerosol model provides a much greater dynamic range for evaluating antivirals. One can choose a low lethal dose of 100 PFU, which is ~3-fold greater than the LD_{50} or use a high dose of 1000 to 10,000 times the LD_{50} to fully test the robustness of the antiviral.

The goal of our initial *in vivo* assessment was to select the two best compounds for a more in depth analysis. A 3 mg/kg dose was chosen for the evaluation as preliminary studies suggested this dose would permit discrimination among the activities of closely related compounds. This drug dose was not toxic as the maximal tolerated dose for HDP-CDV was >30 mg/kg and >10 mg/kg for ODE-, OLP- and OLE-CDV when administered as five consecutive daily doses to uninfected mice. A/NCR mice were treated by gavage with 3 mg/kg of test compounds, exposed to an aerosol dose of 1.7×10^5 PFU of ECTV, and were monitored for mortality, day of death, and lung, spleen and liver infectivity levels (Table 2). As expected from its low bioavailability, CDV failed to protect any mice from a lethal infection, and gave the shortest MTD (9.8±3.5 days) of any of the compounds. ODE-CDV showed the greatest promise among the tested compounds as only 10% of the treated mice died following aerosol challenge, significantly lower virus titers were detected in spleen tissue as compared to the other compounds, and no detectable virus infectivity was found in liver tissue. Careful examination of the survival percentages, MTD

values, and virus titers in tissues indicated that HDP-CDV, OLP-CDV, and OLE-CDV had similar activities. Because the double bond contained in the lipid moiety of OLP-CDV and OLE-CDV could potentially affect long-term drug stability, and since these compounds were not demonstrably better than HDP-CDV, we chose not to study them further *in vivo*.

Dose response evaluation of HDP-CDV and ODE-CDV in an ectromelia virus aerosol challenge model

Groups of A/NCR mice were treated by gavage with 10, 5, 2.5, or 1.25 mg/kg of CDV, HDP-CDV, or ODE-CDV, exposed to an aerosol dose of 2.3×10^4 PFU of ECTV, and monitored for mortality, day of death, and lung, spleen and liver infectivity levels (Fig. 2). As expected, oral CDV failed to provide any protection against a lethal ECTV infection over the range of doses tested. ODE-CDV was clearly more potent than HDP-CDV for protection against a lethal aerosol ECTV challenge as it protected more mice against death at the lower doses (5, 2.5, and 1.25 mg/kg), and also extended the MTD values (Fig. 2, Panel A). Virus titers in lung tissue of HDP-CDV and ODE-CDV treated mice were similar at all tested doses, and only differed significantly from phosphate buffered saline or CDV-treated mice at the 10mg/kg dose (Fig. 2, panel B). Virus titers in spleen tissue of HDP-CDV and ODE-CDV treated mice differed significantly from phosphate buffered saline or CDV-treated mice at all tested doses (Fig. 2, panel C). Although the mean virus titers in spleen tissue of HDP-CDV and ODE-CDV treated mice were similar at doses of 1.25 and 2.5 mg/kg, 3 ODE-treated mice lacked detectable virus infectivity as compared to none for the HDP-CDV treated mice. The greatest difference in tissue virus infectivity between HDP-CDV and ODE-CDV treated mice was observed in the liver, which shows severe pathology in fatal cases of mousepox (Buller & Palumbo, 1991). At the lowest drug dose employed (1.25 mg/kg), virus titers in liver were almost 100-fold lower in ODE-CDV treated mice as compared to the HDP-CDV treatment group (Fig. 2, panel D). In addition, two ODE-CDV treated mice lacked detectable virus infectivity. The apparent difference in liver titers between HDP-CDV and ODE-CDV treated mice at 2.5 mg/kg is not considered significant as each data point contained only one mouse with the remaining 4 mice of each group lacking detectable virus infectivity. When surviving animals were sacrificed at

21 days post-infection at the completion of the experiment, no residual virus infectivity was detected in spleens and livers (data not shown).

Discussion

CDV (Vistide®) is licensed for intravenous use in the treatment of human cytomegalovirus retinitis (HCMV) in HIV-infected patients, and is the subject of a treatment IND for smallpox and complications arising from the smallpox vaccine. It also has broad spectrum antiviral activity against a wide-range of DNA virus and retrovirus infections, but oral bioavailability of <5% hinders its development as an efficacious oral antiviral drug (De Clercq, 1998;Wachsman et al., 1996). Oral bioavailability can be significantly enhanced by derivatization of CDV with ether lipid residues of various chain lengths (Ciesla et al., 2003). Radioactive versions of HDP-, ODE- and OLP-CDV orally administered to mice achieved plasma bioavailability levels of 88 to 97%, and concentrations of 1.4 to 3.4 μM (Ciesla et al., 2003). This enhanced bioavailability appeared due to the general resemblance of alkoxyalkyl esters of CDV to a naturally occurring compound, lysophosphatidylcholine, most of which is absorbed intact from the small intestine (Scow et al., 1967). Unexpectedly, the alkoxyalkyl esters of CDV also showed a dramatic decrease in the EC_{50} values against VACV, CPXV, and HCMV (Kern et al., 2002;Beadle et al., 2002). Using VACV-WR in human foreskin fibroblasts, Kern *et al.* obtained EC_{50} values for HDP-CDV and ODE-CDV of 1.1±1.0 and 0.2±0.2 μM, respectively, which compared favorably with the values summarized in Table 1 (HDP-CDV, 0.5± 0.1 μM and ODE-CDV, 0.2±0.0 μM) (Kern et al., 2002). Importantly the Kern *et al.* study determined the selectivity indices (SI) of HDP-CDV and ODE-CDV to be 6 and 11-fold, respectively, greater than CDV using CPXV and VACV (Kern et al., 2002). The SI value relates the toxicity and efficacy of a compound by dividing the drug's 50% cytotoxic concentration by the 50% effective concentration. As compared to CDV, the larger SI values for analogs HDP-CDV and ODE-CDV indicate that these compound are excellent candidates for *in vivo* efficacy testing.

This marked increase in antiviral activity *in vitro* was due to greatly increased cellular uptake of HDP-CDV relative to CDV. Intracellular levels of the activated antiviral compound, CDV-diphosphate, were 100 times greater after exposure of cells to HDP-CDV than CDV (Aldern et al., 2003). The efficient uptake into cells of alkoxyalkyl esters of CDV such as HDP-CDV is thought to occur via rapid association with cellular membrane phospholipids, whereas the uptake of CDV is slow, and has been shown to occur by fluid phase endocytosis (Connelly et al., 1993). Although this increased cellular uptake of alkoxyalkyl esters of CDV was associated with increased toxicity, the SI index was still significantly better than CDV (Kern et al., 2002). Furthermore, CDV is highly concentrated in the kidney and its dose-limiting toxicity is renal; however, the peak kidney levels of HDP-CDV and ODE-CDV are only 3.3% of CDV (Ciesla et al., 2003). Thus, this class of CDV analogs is worthy of further investigation as possible oral therapies for diseases caused by viruses including HCMV, herpes simplex viruses, and poxviruses.

In this study, we investigated the efficacy of alkoxyalkyl esters of CDV in a mouse model for smallpox. We compared the oral activity of CDV and CDV analogs in an aerosol ECTV challenge model in A/NCR mice. ODE-CDV appeared to be the most potent CDV analog as a 5mg/kg, 5 dose regimen protected 100% of A/NCR mice from a lethal aerosol challenge dose of ~1000 times the LD_{50}. Significant protection was also observed at 1.25mg/kg dose. Virus replication in spleen and liver was not detected when mice were treated with oral doses of ODE-CDV at 5mg/kg or greater; however, virus titers in lung tissue were reduced from control only when the dose was at least 10mg/kg. The relative level of virus infectivity in tissues did not strongly correlate with drug tissue concentration. Peak HDP-CDV and ODE-CDV concentrations, respectively, were 1.00 and 1.19 nmol/g in lung, 0.56 and 0.79 nmol/g in spleen and 43.7 and 25.7 nmol/g in liver (Ciesla et al., 2003). The high levels of HDP-CDV and ODE-CDV in liver tissue may favor antiviral efficacy against ECTV as compared to VACV and VARV since ECTV replicates to far higher levels in liver tissue than does VACV or VARV. The synthesis of an alkoxyalkyl ester of CDV that exerts greater antiviral activity in lung tissue than ODE-CDV, or the administration of aerosolized CDV, may reduce ECTV virus replication in the lung following an aerosolized challenge (Smee et al., 2000; Bray et al., 2002). A strategy of adjusting the character of the lipophilic adduct to increase the concentration of lung CDV-

diphosphate is based on the observation that ^{14}C-labeled ODE-CDV given orally provides substantially higher drug concentration in lung tissue than the slightly less lipophilic HDP-CDV (Ciesla et al., 2003). These approaches may not be necessary, however, as pulmonary disease and presumably extensive variola virus replication in lung was not an early findings in smallpox, rather bronchopneumonia when observed was usually associated with late stage disease (Fenner et al., 1988). A strategy to optimize the pharmacokinetics of alkoxyalkyl esters of CDV would be dependent on a thorough understanding of the pathogenesis of smallpox. This knowledge is currently lacking. The results presented here suggest that the ODE-CDV and similar compounds should be further examined in oral dose optimization and therapeutic intervention studies as potential antivirals against human orthopoxvirus systemic disease caused by VACV, monkeypox virus, and VARV.

Materials and Methods

Cells and virus

BS-C-1 cells (ATCC CCL 26) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine serum fetal clone III (Hyclone, Logan, UT), 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 U/ml penicillin (GIBCO, Grand Island, NY), and 100 µg/ml streptomycin (GIBCO, Grand Island, NY). A plaque-purified isolate of the MOS strain of ECTV (ATCC VR-1374) designated MOS-3-P2, was propagated in an African green monkey kidney cell line, BS-C-1 (Chen et al., 1992). Virus was purified through a sucrose cushion as described elsewhere (Moss & Earl, 1998). The VACV-WR (ATCC VR-119) was propagated as described elsewhere (Moss & Earl, 1998). Virus infectivity was estimated as described previously (Wallace & Buller, 1985). Briefly virus suspensions were serially diluted in PBS +1% sera, absorbed to monolayers for 1 hr at 37° C, and overlayed with a suspension of 1% carboxyl methyl cellulose in DMEM +5 % Fetal clone III. After 4 days at 37° C, virus plaques were visualized and virus inactivated by the addition to each well of 0.5 ml of a 0.3% crystal violet/10% formalin solution.

Animals

Four to six week old female A/NCR mice were obtained from the National Cancer Institute, Frederick Md., housed in filter-top microisolator cages and fed commercial mouse chow and water, *ad libitum*. The mice were housed in an animal biosafety level 3 containment area. Animal husbandry and experimental procedures were in accordance with PHS policy, and approved by the Institutional Animal Care and Use Committee.

Antiviral Compounds

Cidofovir ([S]-1-[3-hydroxy-2-phosphonylmethoxypropyl]cytosine, HPMPC, Vistide®) was purchased from a retail pharmacy or was provided by Gilead Sciences, Inc. (Foster City, CA). Ether lipid analogs of CDV (HDP-CDV, ODE-CDV, OLE-CDV, and OLP-CDE) were prepared as described previously (Beadle et al., 2002). CDV and analogs were prepared fresh for each experiment by dissolving the compounds in sterile, distilled water. Solutions were stored at 4° C over the course of the experiment.

Plaque reduction Assay

CV-1 cells were plated in wells of a 24 well cluster plate. Each monolayer was infected with ~75 plaque forming units (PFU) of indicator virus in 0.1 ml of DMEM +5 % Fetal clone III for 60 min at 37°C. Media was removed by aspiration and standard virus overlay media containing no drug or the test drug at concentrations ranging from 0.05 to 50 μM was added. The plates were incubated at 37°C for 3-4 days for ECTV and 2 days for VACV-WR, monolayers were stained and plaques counted using a stereomicroscope. The EC_{50} concentration for each drug was calculated.

In vivo drug evaluation

Mice were exposed to aerosolized ECTV suspended in MEM using a nose-only inhalation exposure system (NOIES; CH Technologies) equipped with a 1-jet BioAerosol Nebulizing Generator, and operated within a class II biological safety cabinet. The NOIES was operated with a primary air pressure of 20 psi giving 1.5 L/min flow rate to the aerosol chamber (without secondary air), a virus suspension flow rate of 0.5 ml/min, and a system operating pressure~-0.5 in of vacuum relative to the out-side atmospheric pressure. The NOIES delivered a predicted median particle diameter of 0.8 ± 1.2 μm (Dr. Chad Roy, personnel communication). The quantity of virus delivered to the mice over the course of exposure was not measured directly, but estimated by multiplying the concentration of virus in the aerosol (C_A) in PFU by the total volume (V_M) of air respired by a mouse of given body weight over the exposure time using Guyton's formula for minute volumes administered to rodents (Guyton, 1947). This presented virus dose is likely an upper limit as it assumes that all of the virus was optimally aerosolized and completely taken up on inhalation. Four hrs following aerosol exposure to ECTV, groups of 16 mice were treated by gavage with 0.1 ml sterile, distilled water alone or water containing the test compound. This treatment was repeated on days 1, 2, 3, and 4 for a total of five doses. Five mice from each treatment group were sacrificed at 7 days post aerosol challenge, and lung, spleen and liver tissues were isolated. Tissue was ground in PBS (10%w/v), frozen and thawed three times, and sonicated for 20 seconds. Virus infectivity (PFU/ml) in tissue homogenates was estimated by titration on BS-C1 monolayers (above). Arithmetic means were calculated for PFU/ml values above the limit of detection (10^2 PFU/ml). The remaining 11 mice were observed over 21 days for clinical signs of disease (morbidity) and mortality. Mice showing conjunctivitis, little or no movement, and marked respiratory distress were euthanized as they were near death.

Statistical Methods

Statistical significance of differences between groups in the proportion of surviving mice was determined by the Pearson Chi-square test. Statistical significance of differences between groups in tissue infectivity titers was determined by the Wilcoxon non-parametric analysis. $P < 0.05$ was considered significant.

Acknowledgements

R.M.B. was supported by a NIAID contract NO1-AI-15436 and K.Y.H. was supported by NEI grant EY11834, NIAID grant AI29164, and Department of the Army grant DAMD17-01-2-007. The U.S. Army Medical Research Acquisition Activity, Fort Detrick, MD, was the awarding acquisition office. The content of this article does not necessarily reflect the position of policy of the government, and no official endorsement should be inferred.

We would like to thank Dawn Schwartz for editorial assistance and Meribeth Broadway for technical expertise.

References

Aduma, P., Connelly, M.C., Srinivas, R.V., and Fridland, A. 1995. Metabolic diversity and antiviral activities of acyclic nucleoside phosphonates. Mol. Pharmacol. 47, 816-822.

Aldern, K. A., Ciesla, S.L., Winegarden, K.L., and Hostetler, K. Y. 2003. Increased antiviral activity of 1-O-hexadecyloxypropyl-[2-(14)C]cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism. Mol. Pharmacol. 63, 678-681.

Baker, R.O., Bray, M., and Huggins, J.W., 2003. Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections. Antiviral Res. 57, 13-23.

Beadle, J.R., Hartline, C., Aldern, K.A., Rodriguez, N., Harden, E., Kern, E.R., and Hostetler, K., 2002. Alkoxyalkyl esters of cidofovir and cyclic cidofovir exhibit multiple-log enhancement of antiviral activity against cytomegalovirus and herpesvirus replication in vitro. Antimicrob. Agents Chemother. 46, 2381-2386.

Bray, M., Martinez, M., Kefauver, D., West, M., and Roy, C., 2002. Treatment of aerosolized cowpox virus infection in mice with aerosolized cidofovir. Antiviral Res. 54, 129-142.

Bray, M., Martinez, M., Smee, D.F., Kefauver, D., Thompson, E., and Huggins, J.W., 2000. Cidofovir protects

mice against lethal aerosol or intranasal cowpox virus challenge. J. Infec. Dis. 181, 10-19.

Buller, R.M. and Palumbo, G.J., 1991. Poxvirus pathogenesis. Microbiol.Rev. 55, 80-122.

Chen, W., Drillien, R., Spehner, D., and Buller, R. M., 1992. Restricted replication of ectromelia virus in cell culture correlates with mutations in virus-encoded host range gene. Virology 187, 433-442.

Ciesla, S.L., Trahan, J., Wan, W. B., Beadle, J.R., Aldern, K. A., Painter, G.R., and Hostetler, K.Y., 2003. Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney. Antiviral Res. 59, 163-171.

Connelly, M.C., Robbins, B.L., and Fridland, A., 1993. Mechanism of uptake of the phosphonate analog (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) in Vero cells. Biochem.Pharmacol. 46, 1053-1057.

De Clercq, E., 1998. Acyclic nucleoside phosphonates: a new dimension to the chemotherapy of DNA virus and retrovirus infections. J. Med. Microbiol. 47, 1-3.

De Clercq, E., Sakuma, T., Baba, M., Pauwels, R., Balzarini, J., Rosenberg, I., and Holy, A. 1987. Antiviral activity of phosphonylmethoxyalkyl derivatives of purine and pyrimidines. Antiviral Res. 8, 261-272.

Fenner, F., Henderson, D. A., Arita, I., Jezek, Z., and Ladnyj, I.D. Smallpox and its eradication. 1988. Geneva, World Health Organization.

Guyton, A.C., 1947. Measurement of the respiratory volumes of laboratory animals. Am. J. Physiol. 150, 70-77.

Hostetler, K.Y., Beadle, J.R., Kini, G.D., Gardner, M.F., Wright, K.N., Wu, T.H., and Korba, B.A., 1997. Enhanced oral absorption and antiviral activity of 1-O-octadecyl-sn-glycero-3-phospho-acyclovir and related compounds in hepatitis B virus infection, in vitro. Biochem. Pharmacol. 53, 1815-1822.

Kern, E.R., Hartline, C., Harden, E., Keith, K., Rodriguez, N., Beadle, J.R., and Hostetler, K.Y., 2002. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob. Agents Chemother. 46 , 991-995.

Moss, B. and Earl, P.L., 1998. Expression of proteins in mammalian cells using vaccinia viral vectors. Overview of the vaccinia virus expression system. *In* "Current Protocols in Molecular Biology." Vol. 3, p. 16.15.1-16.15.5. John Wiley & Sons, Inc..

Neyts, J. and De Clercq, E., 1993. Efficacy of (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine for the treatment of lethal vaccinia virus infections in severe combined immune deficiency (SCID) mice. J. Med. Virol. 41, 242-246.

Scow, R.O., Stein, Y., and Stein, O., 1967. Incorporation of dietary lecithin and lysolecithin into lymph chylomicrons in the rat. J. Biol. Chem. 242, 4919-4924.

Smee, D.F., Bailey, K.W., Wong, M., and Sidwell, R.W., 2000. Intranasal treatment of cowpox virus respiratory infections in mice with cidofovir. Antiviral Res. 47, 171-177.

Smee, D.F., Bailey, K.W., Wong, M.H., and Sidwell, R.W. 2001. Effects of cidofovir on the pathogenesis of a lethal vaccinia virus respiratory infection in mice. Antiviral Res. 52, 55-62.

Wachsman, M., Petty, B.G., Cundy, K.C., Jaffe, H. S., Fisher, P.E., Pastelak, A., and Lietman, P.S., 1996. Pharmacokinetics, safety and bioavailability of HPMPC (cidofovir) in human immunodeficiency virus-infected

subjects. Antiviral Res. 29, 153-161.

Wallace, G.D. and Buller, R.M. 1985. Kinetics of ectromelia virus (mousepox) transmission and clinical response in C57BL/6j, BALB/cByj and AKR/J inbred mice. Laboratory Animal Science 35, 41-46.

Figure legends

Fig. 1. Structure of cidofovir and ether lipid esters

Fig. 2. Dose response evaluation of HDP-CDV and ODE-CDV in an ectromelia virus aerosol challenge model. Groups of 16 A/NCR mice were treated by gavage with the indicated doses of CDV (■), HDP-CDV (●), or ODE-CDV ( ) on days 0 through 4. Mice were exposed to a small particle aerosol presenting a dose of 2.3 x 10^4 PFU/mouse, which was given~4 hrs **prior** to the first drug treatment. Eleven mice were observed for mortality and MTD (A). Each plotted value is given with its MTD unless no deaths were observed. Five mice from each treatment group were sacrificed at 7 days post challenge, lung (B), spleen (C), and liver (D) tissues were isolated, and virus infectivity measured. Arithmetic means and standard deviation from the means were calculated for PFU/ml titers above the limit of detection in each treatment group. Each tissue sample, which lacked detectable virus (< 10^2 PFU/ml), is represented below the dashed line in each panel. At all treatment doses, the percentage of surviving HDP-CDV and ODE-CDV treated mice differed significantly from that of the CDV treatment group, and at the 1.25 mg/kg dose, the percentage of surviving ODE-CDV treated mice was significantly different from the HDP-CDV treatment group; (P<0.05). In panel B, virus titers in lungs of HDP-CDV and ODE-CDV treated mice differed significantly from the CDV treatment group only at the 10mg/kg dose; (P<0.05). In panel D, virus titers in liver of ODE-CDV treated mice differed significantly from that of the HDP-CDV treatment group only at the 1.25 mg/kg dose; (P<0.05).

In Press, Antimicrobial Agents & Chemotherapy. 10/10/2003

Inhibitory Activity of Alkoxyalkyl and Alkyl Esters of Acyclic Nucleoside Phosphonates Against Orthopoxvirus Replication In Vitro

Kathy A. Keith[1], William B. Wan[2], Stephanie L. Ciesla[2], James R. Beadle[2], Karl Y. Hostetler[2] and Earl R. Kern[1]. [1]Univ. of Alabama School of Medicine, Birmingham, AL, and [2]VA San Diego Healthcare System and the Univ. of California, San Diego, La Jolla, CA 92093-0676

Corresponding Author:
Earl R. Kern, Ph.D.
University of Alabama School of Medicine
Department of Pediatrics
1600 6th Avenue South; CHB 128A
Birmingham, AL 35233
Ph. (205) 934-1990
Fax (205) 975-1992
Email: Kern@uab.edu

Abstract

A new series of ether lipid esters of cidofovir (CDV), cyclic cidofovir (cCDV) were evaluated against vaccinia virus and cowpox virus in tissue culture cells. The results indicated that a number of the compounds were 20 - 600 fold more efficacious than the parent compounds. The antiviral activity of the compounds was strongly dependent on the number of atoms in the chain and the presence or absence of a double bond in the alkoxyalkyl chains linked to the phosphonate moiety of CDV.

The threat of an intentional or unintentional spread of poxvirus infections to a vulnerable population has led to increased efforts to find safe, rapidly deployable treatments against such infections. Although vaccination is now being offered to some healthcare workers and other "first responders", there are valid concerns about potential vaccine risks (Booss and Davis, 2003; Lane and Goldstein, 2003). Previously reported smallpox vaccine-associated adverse reactions, along with the unknown prevalence of risk factors among today's population has prompted the preparation of guidances for clinicians in evaluating and treating patients with smallpox vaccination complications (Cono et al., 2003). Following that guidance, the vaccine is not recommended for those with eczema and other exfoliative skin disorders, those with hereditary or acquired immunodeficiencies, or for pregnant women or women who desire to be pregnant in the 28 days after vaccination. More recently, the Centers for Disease Control issued a health advisory on March 25, 2003 recommending as a precautionary measure, that persons with known cardiac disease not be vaccinated at this time. (CDC Media Relations: Press Release, 2003). Therefore, the use of antiviral therapy in the event of a poxvirus outbreak or in the treatment of vaccination complications against smallpox virus (Bray, 2003), points to the continued need to examine available antiviral therapies, as well as the development of new and more efficient treatment.

The acyclic nucleoside phosphonates Cidofovir (CDV) and cyclic Cidofovir (cCDV) have been shown to be potent inhibitors of poxvirus replication *in vitro* (Baker et al., 2003; Keith et al., 2003; Kern et al., 2002; Snoeck et al., 2002) as well as demonstrating efficacy in animal model studies when administered parenterally or by aerosol (Bray et al., 2000; Quenelle et al., 2003; Smee and Sidwell, 2003) however, these compounds are inactive when given orally. Although CDV is available under Investigational New Drug (IND) protocols for treatment of certain smallpox vaccination complications or for the emergency treatment of smallpox (Cono et al., 2003), the necessity of intravenous hydration and use of probenecid limits its extensive use.

Previous *in vitro* studies have shown that multiple-log increases in antiviral activity were observed with hexadecyloxypropyl and octadecyloxyethyl derivatives of cidofovir and cyclic cidofovir (HDP-CDV; HDP-cCDV, ODE-CDV; ODE-cCDV) against orthopoxvirus replication (Kern et al, 2002), as well as enhanced inhibition of cytomegalovirus and herpesvirus replication (Beadle et al., 2002) by these esters compared to the parent compounds. The compounds have an oral bioavailability of 88 to 93% in mice (Ciesla et al, 2003). Additionally, the oral activity of these cidofovir prodrugs against vaccinia virus (VV) and cowpox virus (CV) infections in mice has been demonstrated (Quenelle et al., 2003).

In this study, the unmodified acyclic nucleoside phosphonates CDV and cCDValong with a new series of analogs synthesized by esterification of these compounds with an alkyl chain without the propoxy linker moiety, were evaluated for efficacy (plaque reduction assay) against VV and CV and cytotoxicity (neutral red uptake assay) in human foreskin fibroblast (HFF) cells using methodologies described previously (Keith et. al., 2003). The compounds were synthesized generally as reported previously (Kern et al, 2002) and the details of the chemistry will be reported elsewhere (Wan, W.B et al, manuscript in preparation, 2003).

As presented in Table 1, the most active ether lipid esters of CDV were OLE-CDV, ODBG-CDV, TDP-CDV, OLP-CDV and ODP-CDV with 50% effective concentrations (EC_{50}s) of 0.06 – 1.2 μM for VV and 0.07 – 1.9 μM for CV. These analogs demonstrated 20 – 600 fold increases in

efficacy when compared to the parent compound. Comparisons of these CDV analogs with published results of *in vitro* activity of HDP-CDV and ODE-CDV (Kern et al., 2002), as well as additional testing of these compounds reported in this study, indicated that several of the newly synthesized compounds were even more efficacious than HDP-CDV or ODE-CDV, which had increases up to 200-fold over the parent compound. Most of the cCDV analogs followed a comparable but lower pattern of efficacy when compared to their CDV counterparts, with 3 – 150 fold increases over the parent compound cCDV. The selectivity index (SI) value, which measures a compounds activity by evaluating the toxicity (CC_{50}) divided by efficacy (EC_{50}), ranged from 85 to 933 for OLE-CDV; OLE-cCDV and OLP-CDV; OLP-cCDV against both viruses. Comparatively, SI values for HDP-CDV; HDP-cCDV and ODE-CDV; ODE-cCDV were in the 40 – 140 range.

The majority of the new analogs tested were more active than the parent compounds; only 4 CDV and 2 cCDV analogs were inactive up to 20 μM. The less active compounds were alkoxyalkyl or alkyl esters of CDV having short chains of less than 16 atoms beyond the phosphonate moiety of CDV. Interesting new analogs were tetradecyloxypropyl-CDV which was equally active with HDP-CDv but with a much higher SI. Figure 1 shows the comparative activity of the CDV derivatives with no linker and with the propyxy linker. CDV esters of this type have rather sharp optimum chain lengths of 18 to 20 atoms beyond the phosphonate and there is little difference between their activity when the are compared in this way. Cyclic CDV analogs show a sharp optimum in antiviral activity at 20 atoms with activity declining sharply as the chain is lengthened to 22 atoms. Interestingly, tetradecyloxypropyl-CDV is substantially less cytotoxic but retains excellent antiviral activity and selectivity, SI >200. Addition of a 9,10 cis double bond into the eighteen carbon alkyl chain (oleyl-) substantially increased the activity about 3 fold relative to the saturated alkyl chain. The oxyethyl analogs of CDV are generally more active than their oxypropyl counterparts even though they differ by only one methylene. OLE-CDV was the most active and selective derivative of CDV in these studies with an EC50 of

0.06 and a SI of 933 against vaccinia and and EC50 of 0.07 and an SI of 800 versus cowpox (Table 1).

Several of these new analogs have enhanced activity against orthopoxvirus replication *in vitro*, and warrant further investigation for determining their oral absorption and efficacy in animal models.

These studies were supported by contract NO1-AI-85347 from the Antiviral Research Branch, NIAID, NIH to the University of Alabama at Birmingham (ERK) and by NIH grants EY11832 and AI21694 to the San Diego Veterans Medical Research Foundation and Department of Defense grant DAMD17-01-2-0071 from the US Army Medical Research and Material Command (KYH).

References

Baker, R.O., Bray, M. and J.W. Huggins. 2003. Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections. Antiviral Res. **57**:13-23.

Beadle, J.R., Hartline, C., Aldern, K.A., Rodriguez, N., Harden, E., Kern, E.R. and K.Y. Hostetler. 2002. Alkoxyalkyl esters of cidofovir and cyclic cidofovir exhibit multiple-log enhancement of antiviral activity against cytomegalovirus and herpesvirus replication in vitro. Antimicrob. Agents Chemother. **46**:2381-2386.

Booss, J. and L.E. Davis. 2003. Smallpox and smallpox vaccination. Neurological implications. Neurol. **60**:1241-1245.

Bray, M., Martinez, M., Smee, D.F., Kefauver, D., Thompson, E. and J.W. Huggins. 2000. Cidofovir protects mice against lethal aerosol or intranasal cowpox virus challenge. J. Infect. Dis. **181**:10-19.

Bray, M. 2003. Pathogenesis and potential antiviral therapy of complications of smallpox vaccination. Antiviral Res. **58**:101-114.

Cono, J., Casey, C.G. and D.M. Bell. 2003. Smallpox vaccination and adverse reactions. Guidance for clinicians. MMWR Recomm Rep. **52**:1-28.

Ciesla, S.L., Trahan, J., Winegarden, K.L., Aldern, K.A., Painter, G.R. and Hostetler, K.Y. 2003. Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney, Antiviral Res. **59**: 163-171.

De Clercq, E., Sakuma, T., Baba, M., Pauwels, R., Balzarini, J., Rosenberg, I. and A. Holý. 1987. Antiviral activity of phosphonylmethoxyalkyl derivatives of purine and pyrimidines. Antiviral Res. **8**:261-272.

De Clercq, E. 2001. Vaccinia virus inhibitors as a paradigm for the chemotherapy of poxvirus infections. Clin. Microbiol. Rev. **14**:382-397.

Holý, A., Günter, J., Dvořáková, H., Masojídková, M., Andrei, G., Snoeck, R., Balzarini, J. and E. De Clercq. 1999. Structure-antiviral activity relationship in the series of pyrimidine and purine N-[2-(2-phosphonomethoxy)ethyl] nucleotide analogues. 1. Derivatives substituted at the carbon atoms of the base. J. Med. Chem. **42**:2064-2086.

Keith, K.A., Hitchcock, M.J.M., Lee, W.A., Holý, A. and E.R. Kern. 2003. Evaluation of nucleoside phosphonates and their analogs and prodrugs for inhibition of orthopoxvirus replication. Antimicrob. Agents Chemother. **47**:2193-2198.

Kern, E.R., Hartline, C., Harden, E., Keith, K., Rodriguez, N., Beadle, J.R. and K.Y. Hostetler. 2002. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob. Agents Chemother. **46**:991-995.

Kern, E.R. 2003. In vitro activity of potential anti-poxvirus agents. Antiviral Res. **57**:35-40.

Lane, J.M. and J. Goldstein. 2003. Evaluation of 21st-century risks of smallpox vaccination and policy options. Ann. Intern. Med. **138**:488-493.

Quenelle, D.C., Collins, D.J. and E.R. Kern. 2003. Efficacy of multiple and single dose cidofovir in vaccinia and cowpox virus infections of mice. Antimicrob. Agents Chemother. In press.

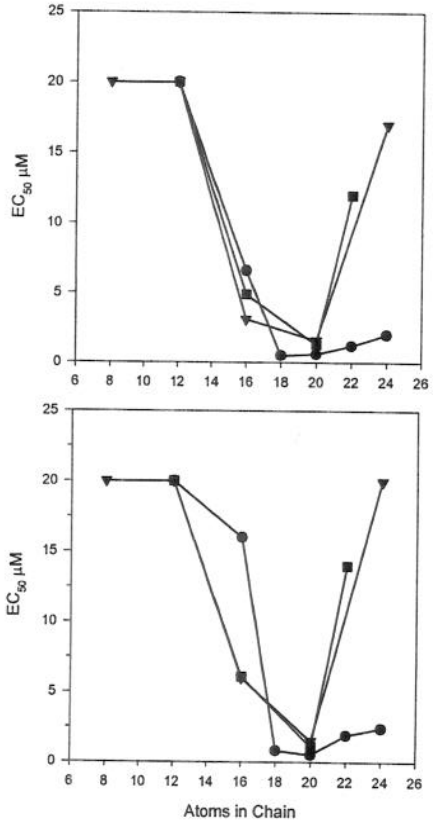
Quenelle, D.C., Collins, D.J., Wan, W.B., Beadle, J.R., Hostetler, K.Y and E.R. Kern,. 2003. Oral treatment of cowpox or vaccinia virus infections in mice with ether lipid esters of cidofovir. Antimicrob. Agents Chemother. In press.

Smee, D.F. and R.W. Sidwell. 2003. A review of compounds exhibiting anti-orthopoxvirus activity in animal models. Antiviral Res. **57**:41-52.

Snoeck, R., Holý, A., Dewolf-Peeters, C., Van Den Oord, J., De Clercq, E. and G. Andrei. 2002. Antivaccinia activities of acyclic nucleoside phosphonate derivatives in epithelial cells and organotypic cultures. Antimicrob. Agents Chemother. **46**:3356-3361.

Figure 1. Effect of Alkyl or Alkoxyalkyl Chain Length on the Antiviral Activity of CDV and cyclic CDV analogs against vaccinia and cowpox, in vitro

FIGURE 1 REVISED



Top panel: EC_{50} versus Vaccina; Bottom panel: EC_{50} versus Cowpox

Symbols: Circles, CDV-propyloxyl-R; Triangles, CDV-no linker-R; Squares, cyclic CDV-propyloxy-R; where R is an alkyl chain

Table 1. Efficacy and Cytotoxicity of Ether Lipid Esters of Cidofovir (CDV) and cyclic Cidofovir (cCDV)

[Revised]

			Vaccinia Copenhagen		Cowpox Brighton	
Compound	**Abbreviation**	**CC_{50} (μM)[a]**	**EC_{50} (μM)[a]**	**SI[b]**	**EC_{50} (μM)[a]**	**SI[b]**
Cidofovir Series						
Cidofovir	CDV	>317 ± 0	31 ± 5.4	>10	42 ± 5.4	>7.5
Propanediol linkers						
Octyloxypropyl- (12)[c]	OP-CVD	80 ± 32	>20 ± 0	--	>20 ± 0	--
Dodecyloxypropyl- (16)	DDP-CDV	>190 ± 0	6.6 ± 0.9	>29	16 ± 1.6	>12
Tetradecyloxypropyl- (18)	TDP-CDV	>100 ± 0	0.5 ± 0.2	>200	0.8 ± 0.5	>125
Hexadecyloxypropyl- (20)	HDP-CDV	29 ± 2.3	0.6 ± 0.4	48	0.5 ± 0.3	58
Octadecyloxypropyl- (22)	ODP-CDV	44 ± 14	1.2 ± 0.5	37	1.9 ± 0.6	23
Oleyloxypropyl- (22:1)	OLP-CDV	87 ± 15	0.4 ± 0.2	218	0.6 ± 0.3	145
Eicosyloxypropyl- (24)	ECP-CDV	92 ± 1.4	2.0 ± 0.9	46	2.4 ± 0.7	38
Ethanediol linkers						
Octadecyloxyethyl- (21)	ODE-CDV	21 ± 8.8	0.2 ± 0.1	105	0.2 ± 0.2	105
Oleyloxyethyl- (21:1)	OLE-CDV	56 ± 29	0.06 ± 0.02	933	0.07 ± 0.02	800
Glycerol linker						
1-O-Octadecyl-2-O-benzyl-glyceryl-	ODBG-CDV	47 ± 24	0.4 ± 0.1	118	0.3 ± 0.01	157
No Linker						
Octyl- (8)	O-CDV	>100	>20 ± 0	--	>20 ± 0	--
Dodecyl- (12)	DD-CDV	>100	>20 ± 0	--	>20 ± 0	--
Hexadecyl- (16)	HD-CDV	>157 ± 58	3.1 ± 0.1	>51	6.0 ± 2.0	>26
Eicosyl- (20)	EC-CDV	45 ± 8.5	1.6 ± 1.3	28	1.5 ± 0.9	30
Tetracosyl- (24)	TC-CDV	>100	>17 ± 4.2	--	>20 ± 0	--
Cyclic Cidofovir Series						
Cyclic Cidofovir	cCDV	>331 ± 0	37 ± 10	>9.0	45 ± 9.4	>7.4
Octyloxypropyl- (12)	OP-cCDV	>84 ± 16	>20 ± 0	--	>20 ± 0	--
Dodecyloxypropyl- (16)	DDP-cCDV	63 ± 2.7	4.9 ± 2.7	13	6.1 ± 2.7	10
Hexadecyloxypropy- (20)	HDP-cCDV	17 ± 0.1	1.3 ± 1.0	13	1.1 ± 0.5	16
Octadecyloxypropyl- (22)	ODP-cCDV	77 ± 3.7	12 ± 1.1	6.4	14 ± 4.2	5.5
Oleyloxypropyl- (22:1)	OLP-cCDV	46 ± 0.6	0.5 ± 0.1	92	0.4 ± 0.1	115
Octadecyloxyethyl- (21)	ODE-cCDV	44 ± 8.0	0.3 ± 0.1	147	0.4 ± 0.1	110
Oleyloxyethyl- (21:1)	OLE-cCDV	34 ± 11	0.4 ± 0.02	85	0.3 ± 0.06	113
1-O-Octadecyl-2-O-benzyl-glyceryl-	ODBG-cCDV	77 ± 32	8.6 ± 1.5	9.0	5.8 ± 3.7	13
Hexadecyl-	HD-cCDV	83 ± 20	>20 ± 0	--	>20 ± 0	--

Values are the mean of 2 or more assays ± standard deviation.. b. Selectivity Index (SI) = CC_{50}/EC_{50} ; c. Values in parentheses are the number of atoms beyond the phosphonate oxygen; the number after the colon is the number of double bonds in the alkyl chain.

In Press

Oral Activity of Ether Lipid Ester Prodrugs of Cidofovir Against Experimental Human Cytomegalovirus Infections

Running Title: Efficacy of oral cidofovir prodrugs against HCMV

By

Deborah J. Bidanset[1], James R. Beadle[2], W. Brad Wan[2], Karl Y. Hostetler[2], and Earl R. Kern[1*]

[1]University of Alabama School of Medicine, Birmingham, AL 35233 and [2] San Diego VA Healthcare System and University of California, San Diego, La Jolla, CA 92093

*Corresponding Author
Earl R. Kern, PhD
The University of Alabama at Birmingham
Department of Pediatrics
128 Children's Harbor Building
1600 6th Avenue South
Birmingham, AL 35233
Tel: (205) 934-1990
Fax: (205) 975-1992
E-mail: kern@uab.edu

Abstract

Infection with human cytomegalovirus (CMV) can cause serious complications in bone marrow and solid organ transplant patients and current therapies are not optimal. We have evaluated two orally active ether lipid ester analogs of cidofovir (CDV), hexadecyloxypropyl-cidofovir (HDP-CDV) and octadecyloxyethyl-cidofovir (ODE-CDV), in severe combined immunodeficient mice in which fetal retina or thymus and liver tissue was implanted and later infected with CMV. The results indicated that oral HDP-CDV or ODE-CDV treatment was 4 to 8 fold more active on a molar basis than intraperitoneal CDV. These data suggest that HDP-CDV and ODE-CDV should be evaluated further as potential antiviral agents for CMV infections.

Introduction

Cytomegalovirus (CMV) infection continues to be a major cause of morbidity in stem cell and solid organ transplant patients [1] and is reported to be the most frequent congenital infection worldwide occurring in 0.2-2.5% of all live births [2]. There are five therapeutics currently approved for treatment of CMV but in many cases, long term therapy is ineffective, may result in the development of resistance or serious adverse events that require termination of treatment [3]. These problems clearly indicate the need for better antiviral agents against CMV.

One of the more active drugs for CMV infection is cidofovir (CDV), however, lack of oral activity requires administration via intermittent intravenous infusion. To produce orally active analogs of CDV, two ether lipid ester prodrugs, hexadecyloxypropyl-cidofovir (HDP-CDV) and octadecyloxyethyl-cidofovir (ODE-CDV) were synthesized [4, 5]. In vitro evaluation of these compounds indicated that both were 100-10,000 times more active against various laboratory, clinical and drug-resistant isolates of CMV than CDV or ganciclovir [5]. This increased activity has been attributed, at least for HDP-CDV, to enhanced cellular uptake as well as increased intracellular levels of CDV diphosphate when compared to CDV [6]. Pharmacokinetic evaluation of the two analogs of CDV in mice indicate that oral bioavailability of HDP-CDV and ODE-CDV was 93% and 88%,respectively, compared with CDV at <5% [7]. This excellent bioavailability combined with an observation that accumulation of these analogs in the kidney was markedly reduced suggested that HDP-CDV or ODE-CDV could be more conveniently administered and may be less nephrotoxic than CDV.

In these studies, we utilized two animal models of human CMV infection to evaluate the oral efficacy of HDP- and ODE-CDV. In these models, either fetal human

retinal or thymus and liver tissue was implanted into severe combined immunodeficient (SCID) mice and later infected with CMV. Animals were treated with either vehicle, HDP-CDV or ODE-CDV and at various times after infection, implanted tissue was removed and CMV titers quantified by plaque assay.

Materials and Methods

Antiviral Drugs

Cidofovir (CDV; Vistide®) was provided by Gilead Pharmaceuticals (Foster City, CA) and the ether lipid esters, HDP-CDV and ODE-CDV were synthesized as described previously [4, 5]. Compounds were diluted in sterile saline to provide the desired dose in either 0.1 ml (i.p.) or 0.2 ml (p.o.).

Preparation of Viral Pools

The Toledo strain of CMV was obtained from Dr. Edward Mocarski (Stanford University School of Medicine, Stanford, CA, USA) and propagated in human foreskin fibroblast (HFF) cells derived from primary cultures [8, 9].

Implantation and Infection of Retinal Implants

Implantation of human fetal retinal tissue and later infection of the retinal tissue implants was performed as described elsewhere [8, 10]. Briefly, 4-8-week-old male SCID mice (Charles River Laboratories, Raleigh, NC, USA) were anesthetized with an i.p. injection of 100 mg/kg ketamine and 15 mg/kg xylazine and proparacaine-HCl (0.5%) was instilled in the eyes. A winged infusion needle (27G X ½") containing mechanically dissociated fetal retinal tissue in Optisol (Chiron Ophthalamics, Irvine, CA, USA) was then inserted into the anterior chamber of the mouse eye where approximately 5 µl of tissue was injected. Using a similar procedure, mice were again anesthetized 8-12 weeks later and 10 µl of virus (4-5000 pfu) was injected near the implant. Beginning 24 h after infection, once daily treatment of mice was initiated and continued for 28 days.

To monitor CMV replication in retinal tissue implants, approximately 6 mice from each group were euthanized at 7, 14, 21, and 28 days after infection. The eyes were

removed and homogenized in 1.0 ml of MEM containing 10% FBS, 2 mM L-glutamine, 200 U/ml penicillin, 50 µg/ml gentamicin and 3 µg/ml fungizone. The homogenate was centrifuged and the supernatant removed and frozen at -70°C until assayed for CMV.

Implantation and infection of Thymus/Liver Implants

Implantation and later infection of thymus/liver (thy/liv) tissue under the kidney capsule of SCID mice was performed as described previously [9]. In these experiments, 4-8 week-old male SCID mice were anesthetized and fragments of fetal thymus and liver were implanted under the kidney capsule using an 18 gauge trocar. After recovery, mice were given 0.8 mg sulfamethoxazole and 0.16 mg trimethoprim per ml in drinking water for two weeks.

Implants were allowed to develop for 12-16 weeks before being inoculated with 6-7000 pfu of CMV. Beginning 24 h after infection, treatment was initiated and continued once daily for 28 days. On days 14, 21, 28, and 35, approximately 10 implants in each group were biopsied (~50% of implant), weighed and homogenized in MEM. Homogenates were centrifuged and supernatants removed and frozen at -70°C until assayed for virus.

Statistical Evaluation

As data were not normally distributed and there were a number of zero values in several of the data sets, we used a non-parametric stratified Wilcoxon Rank sum test to evaluate differences in virus replication between vehicle and drug-treated mice. Days were used as strata and data collected during the entire 28 days of treatment were used.

Results

Several experiments were performed initially to determine a maximal tolerated dose and the minimal effective dose of the analogs. In the first experiment, SCID-human (SCID-hu) retinal tissue implants were used and the results indicated that daily treatment with 20 mg CDV/kg, 10 mg HDP-CDV/kg or 5 mg HDP-CDV/kg effectively reduced replication of CMV (data not shown). In a second experiment, SCID-hu thy/liv implants were utilized and those results indicated that in comparison to the vehicle-treated group, treatment with either 10 mg CDV/kg or 10, 5, or 2.5 mg HDP-CDV/kg was also effective in inhibiting the replication of human CMV (data not shown). To determine the in vivo activity of ODE-CDV, another series of SCID-hu thy/liv implants were infected and treated orally with vehicle or 5, 2.5, or 1.25 mg ODE-CDV/kg, or i.p. with 20 mg CDV/kg. These results indicated that treatment with either CDV or ODE-CDV was effective in inhibiting the replication of human CMV in a dose dependent manner (data not shown).

In the experiments presented here, a direct comparison between i.p. CDV and oral HDP-CDV or ODE-CDV was made in both SCID-hu retinal and thy/liv implant models. In the first of these, SCID-hu retinal tissue implants were infected with 4700 pfu of CMV per eye. Beginning 24 h after infection, animals were treated once daily orally with either vehicle (saline), 10 or 5 mg HDP-CDV/kg, 5 or 2.5 mg ODE-CDV/kg, or i.p. with 20 mg CDV/kg. Treatment was continued for 28 days and on days 7, 14, 21, 28, animals were euthanized, eyes removed, homogenized, and assayed for CMV. The results indicated that, compared to the vehicle control, treatment with CDV, HDP-CDV or ODE-CDV reduced significantly ($p<0.0001$) replication of CMV in the implant tissue (Figure 1). By 21 days after infection, the mean viral titer in implants from the vehicle

group was 4.3 $\log_{10}$ pfu/g. In comparison, mean viral titer from implants in the CDV-treated group was 1.9 $\log_{10}$ pfu/g. In mice treated with 5 mg HDP-CDV/kg or 2.5 mg ODE-CDV/kg, mean viral titers were 1.9 and 2.4 $\log_{10}$ pfu/g, respectively. Importantly, in implants from animals treated with either 10 mg HDP-CDV/kg or 5 mg ODE-CDV/kg, no virus was detected.

In a final comparison between the three compounds, thy/liv implant tissue was infected with 7000 pfu of CMV. Beginning 24 h later, animals were treated and tissue harvested using a regimen similar to that stated above. The results, in Figure 2, indicated that compared to titers obtained in tissues from vehicle treated mice, treatment with CDV, HDP-CDV, or ODE-CDV were all effective ($p<0.001$) in inhibiting CMV replication. By 28 days after infection, the mean viral titer in vehicle-treated animals was 5.7 $\log_{10}$ pfu/g. In comparison, mean viral titers in implant tissue from animals treated with either 20 mg CDV/kg or 2.5 mg ODE-CDV/kg were 3.3 and 2.6 $\log_{10}$ pfu/g, respectively. In animals treated with either 10 or 5 mg HDP-CDV/kg or 5 mg ODE-CDV/kg, again, no infectious virus was detectable.

During the course of several of the above experiments, animals were weighed on a thrice weekly basis. In general, animals lost approximately 1-2% of their original body weight by 4 days but gained back and maintained their weight for the remainder of the treatment period. On the last day of treatment, blood chemistries were performed and most of the values obtained were similar to those from vehicle-treated or CDV-treated animals (data not shown).

Discussion

In these studies, we have demonstrated that orally administered HDP-CDV and ODE-CDV are highly effective against CMV infection in vivo. In each experiment, both compounds administered orally were found to be 4 to 8 fold more efficacious on a molar basis than intraperitoneal CDV. In the SCID-hu thy/liv model, the activity of HDP-CDV and ODE-CDV indicated that oral administration of these compounds results in drug levels sufficient to inhibit CMV replication in target organs. In the SCID-hu retinal model, the efficacy of these compounds suggested that in addition to systemic activity, HDP-CDV and ODE-CDV can cross the blood-ocular barrier and inhibit CMV infection of ocular tissue as well.

These data further support the SCID-hu retinal tissue and thy/liv models for evaluating various antiviral therapies against both ocular and systemic CMV infections in vivo. Studies validating the use of these models for the evaluation of antiviral therapies have shown that while they do not precisely mimic human CMV infections, results after treatment with GCV or CDV [8, 9, 11] appear to correlate with clinical outcome [3].

In addition to activity against CMV, HDP-CDV and ODE-CDV are also highly efficacious against other herpesviruses in vitro [12]. The activity of these compounds was typically shown to be 2-3 logs more efficacious against herpes simplex virus types 1 and 2, Epstein Barr virus and human herpesviruses 6 and 8 than that observed for CDV [12]. We have also reported the enhanced in vitro activity of these compounds over CDV against vaccinia and cowpox virus [4]. Further evaluation of these compounds against orthopoxvirus infection in vivo confirmed that both HDP-CDV and

ODE-CDV were as effective as CDV in inhibiting viral replication and preventing mortality in mice inoculated intranasally with these viruses [13].

It is interesting to note that while HDP-CDV and ODE-CDV were significantly more efficacious than CDV in vitro against vaccinia and cowpox viruses, the in vivo efficacy of these orally administered compounds did not appear to be significantly different from parenterally administered CDV [13]. The HDP- and ODE- analogs of CDV exhibited a 2.5-4 log increase in activity against various isolates of human CMV in vitro [5]. In contrast, the present studies indicate that the oral activity of HDP-CDV and ODE-CDV is only 4 to 8-fold more active than parenteral CDV in inhibiting CMV replication. These results can partially be explained in the SCID-hu thy/liv model, where tissue implantation and infection occur under the kidney capsule, by pharmacokinetic data which indicates that the $AUC_{0\text{-}72h}$ levels of HDP-CDV and ODE-CDV in kidney are 20-25% of those observed for CDV [7]. Thus, parameters such as in vitro efficacy, bioavailability and tissue levels of drugs all must be considered together when interpreting the potential of these compounds against various viral infections.

HDP-CDV and ODE-CDV are orally bioavailable compounds that have enhanced activity against CMV in vitro (5). In vivo, these studies indicate that orally administered HDP-CDV and ODE-CDV are 4 to 8 fold more active on a molar basis than parenteral CDV. After oral administration of these analogs, plasma and tissue levels of CDV were higher and persisted longer in all tissues examined with the exception of kidney when compared to parenteral CDV [7]. These observations suggest that oral HDP-CDV and ODE-CDV may result in less nephrotoxicity than seen with CDV by minimizing accumulation of drug in kidney. The data presented in this

report strongly suggests that HDP-CDV and ODE-CDV are excellent candidates for further evaluation in the treatment of CMV as well as other DNA virus infections.

Acknowledgements

These studies were supported by contracts NO1-AI-15439 and NO1-AI-65290 and grant PO1-AI-46390 from NIAID, NIH to the University of Alabama at Birmingham and by NIH grants EY11832 and AI21694 to the San Diego Veterans Medical Research Foundation and Department of Defense grant DAMD 17-01-2-0071 from the US Army Medical Research and Material Command. We thank Dr. Debra Quenelle, Deborah Collins and Bridgett Herrod for their invaluable technical support and Dr. Sreelatha Meleth (UAB School of Medicine, Birmingham, AL, USA) for statistical support.

References

1. Ljungman P. β-Herpesvirus challenges in the transplant recipient. J Infect. Dis. **2002**; 186 (Suppl. 1): S99-109.
2. Leung AKC, Sauve RS, and Davies HD. Congenital Cytomegalovirus Infection. J Natl Med Assoc **2003**; 95: 213-218.
3. Jacobsen MA. Drug therapy: treatment of cytomegalovirus retinitis in patients with acquired immunodeficiency syndrome. N Engl J Med **1997**; 337: 105-14.
4. Kern ER, Hartline C, Harden E, Keith K, Rodriguez N, Beadle JR, and Hostetler KY. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cydofovir. Antimicrob Agents Chemother **2002**; 46: 991-5.
5. Beadle JR, Hartline C, Aldern KA, Rodriguez N, Harden E, Kern ER, and Hostetler KY. Alkoxyalkyl esters of cidofovir and cyclic cidofovir exhibit multiple log enhancement of antiviral activity against cytomegalovirus and herpesvirus replication in vitro. Antimicrob Agents Chemother **2002**; 46: 2381-6.
6. Aldern KA, Ciesla SL, Winegarden KL, and Hostetler KY. Increased antiviral activity of 1-O-hexadecyloxypropyl-[2-^{14}C]cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism. Mol Pharmacol **2003**; 63: 678-81.
7. Ciesla SL, Trahan J, Wan WB, Beadle JR, Aldern KA, Painter GR, and Hostetler KY. Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes drug accumulation in kidney. Antivir Res **2003**; 59: 163-71.
8. Bidanset DJ, del Cerro M, Lazar E, Faye-Petersen O, and Kern ER. Animal model for ocular human cytomegalovirus infections in SCID-hu mice. In: Zak O

and Sande ME, eds. Handbook of Animal Models of Infection. New York: Academic Press **1999**: 957-62.

9. Kern ER, Rybak RJ, Hartline CV, and Bidanset DJ. Predictive efficacy of SCID-hu mouse models for treatment of human cytomegalovirus. Antivir Chem Chemother **2001**; 12 Suppl 1: 149-56.
10. Bidanset DJ, Rybak RJ, Hartline CB, and Kern ER. Replication of human cytomegalovirus in severe combined immunodeficient mice implanted with human retinal tissue. J Infect Dis **2001**; 184: 192-5.
11. Mocarski ES, Bonyhadi M, Salimi S, McCune JM and Kaneshima H. Human cytomegalovirus in a SCID-hu mouse: thymic epithelial cells are prominent targets of viral replication. Proc Natl Acad Sci USA **1993**; 90: 104-8.
12. Williams SL, Kushner NL, Hartline CB, Harden EA, Beadle JR, and Hostetler KY. Enhanced activity of alkoxyalkyl esters of cidofovir and cyclic cidofovir against replication of herpesviruses in vitro [abstract 89]. Antivir Res **2002**; 53: A61.
13. Quenelle DC, Collins DJ, Wan WB, Beadle JR, Hostetler KY, and Kern ER. Oral treatment of cowpox and vaccinia virus infections in mice with ether lipid esters of cidofovir. Antimicrob Agents Chemother **2003**; in press.

Figures

Figure legends

Figure 1 The effect of oral HDP- and ODE-CDV treatment on the replication of human CMV in SCID-hu retinal tissue implants. Dosages are mg/kg

Figure 2 The effect of oral HDP- and ODE-CDV treatment on the replication of human CMV in SCID-hu thy/liv tissue implants. Dosages are in mg/kg.

Figure 1

The Effect of Oral HDP- and ODE-CDV Treatment on the Replication of HCMV in SCID-hu Retinal Tissue Implants

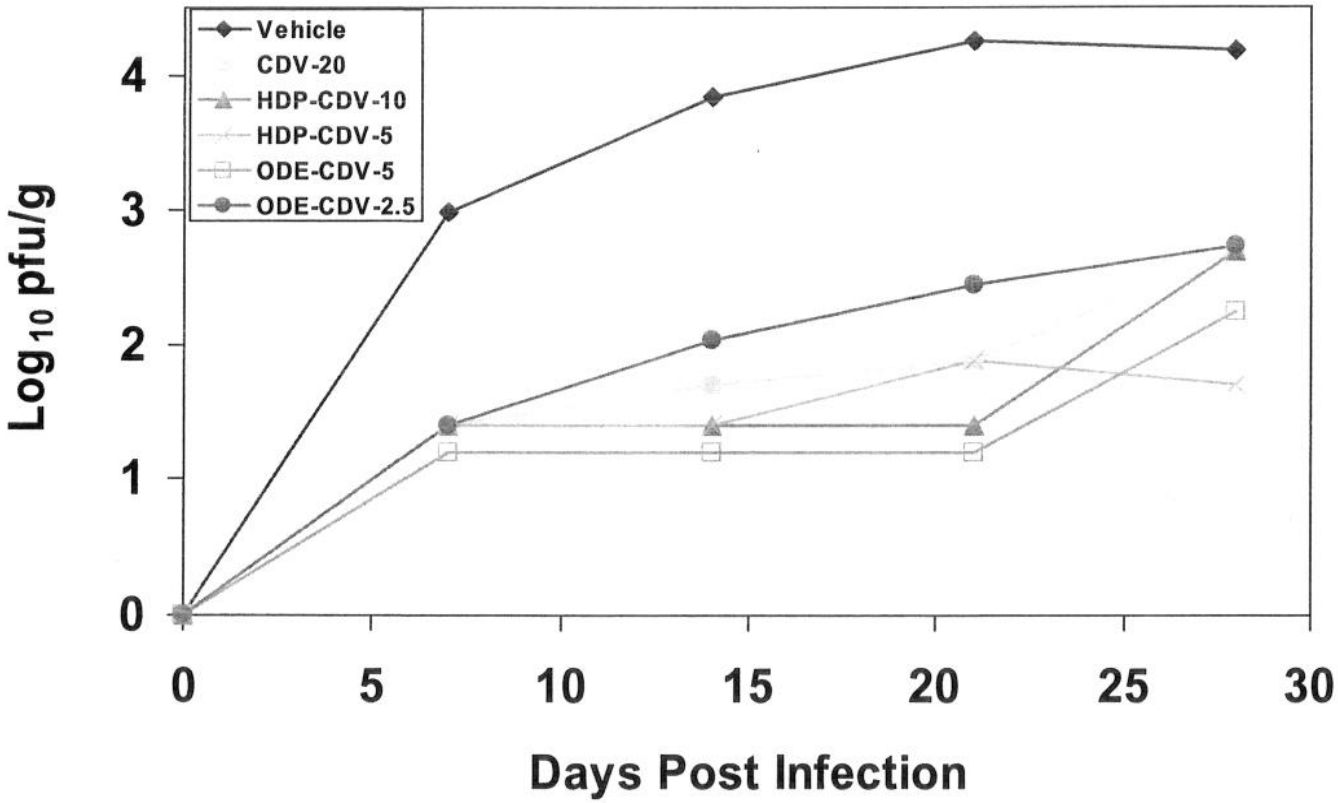


Figure 2

The Effect of Oral HDP- and ODE-CDV Treatment on the Replication of HCMV in SCID-hu Thy/Liv Tissue Implants